Neural Stem (NS) cells, from Mouse, Rat, and Human

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Abbreviations

BLBP	Brain Lipid Binding Protein
BDNF	Brain Derived Neurotrophic Factor
BMP	Bone Morphogenetic Protein
CNS	Central Nervous System
EBs	Embryoid Bodies
EGF	Epidermal Growth Factor
ES cell	Embryonic Stem cell
FACS	Fluorescent Activated Cell Sorting
FGF2	Fibroblast Growth Factor 2
GFP	Green Fluorescent Protein
GFAP	Glial Fibrillary Acidic Protein
GLAST	Astrocyte-specific Glutamate Transporter
LIF	Leukemia Inhibitory Factor
MAP2	Microtubule-associated Protein 2
MAPK	Mitogen Activated Protein Kinase
NS cell	Neural Stem Cell
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
RMS	Rostral Migratory Stream
SGL	Subgranular Layer
Shh	Sonic Hedgehog
SVZ	Subventricular Zone
TGF	Transforming Growth Factor

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Abstract

Neural stem cells are the self-renewing and oligopotent cell population that generate constituent cell types of the nervous system. Cultured neural stem cells would offer researchers accessible opportunities to answer fundamental questions in both neurodevelopment and cell biology. Current strategies of maintaining neural stem/progenitor cells *in vitro* largely rely on neurosphere cultures (Reynolds and Weiss, 1992) and/or genetic immortalization (Frederiksen et al., 1988; Sah et al., 1997), These approaches raise concerns about cellular heterogeneity and potential cell transformation. Our lab has recently reported the establishment of adherent mouse Neural Stem (NS) cell lines that undergo symmetrical self-renewal without genetic immortalization (Conti et al., 2005; Pollard et al., 2006). Here, I apply this approach to human and rat foetal tissue and describe the derivation and characterization of human and rat NS cell lines.

I established Human foetal NS cell lines from elective termination tissue. Human NS cells are propagated as stable cell lines in the presence of both epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2), under which conditions they stably express neural precursor markers and exhibit negligible differentiation into neurons or glia. Human NS cells are induced to produce astrocytes, oligodendrocytes, and mature neurons upon exposure to appropriate differentiating conditions. Human NS cells are clonogenic stem cells. They are capable of generating clonal and tripotent cell lines from single deposited cells, demonstrating they represent self-renewing *in vitro* human neural stem cell populations. More importantly, human NS cells retain a diploid karyotype and constant neurogenic capacity for more than 100 generations, and their long-term stability does not require leukemia inhibitory factor (LIF). Together with the demonstrations that human NS cells can be genetic modified and are accessible to multi-well time-lapse videomicroscopy, these cells creates the potential for high content genetic and chemical screens.

In addition to human foetal tissue, adherent NS cells can also be derived from rat foetal brain and spinal cord. However, under standard expansion conditions supplemented with EGF and FGF2 (Conti et al. 2005), rat NS cells spontaneously become dormant after approximately 2 months expansion. Dormant rat cells exhibit stellate morphology and express the astroglial maker GFAP, but they still retain neural precursor makers such as Nestin and Sox2. I found that Bone Morphogenetic Protein (BMP) signals are responsible for generating quiescence of rat NS cells, and that FGF2 signaling inhibits BMP-induced astrocyte differentiation and therefore maintains stem cell potency. Applying NS cell conditioned medium or BMP antagonist Noggin could overcome cell quiescence, and by these means the long-term propagation of rat foetal NS cells can be maintained. In addition to foetal NS cells, Noggin also promotes the proliferation of adult rat subventricular zone (SVZ) neural precursors. These observations implies that the neurogenic but quiescent rat NS cells generated by BMP and FGF2 signals may reflect some characteristics of *in vivo* adult neural stem cells.

Lastly, I undertake preliminary investigation of intracerebral transplantation using established NS cell lines. Mouse NS cells labelled with green fluorescent protein (GFP) were injected into cortex, striatum and hippocampus of both adult and neonatal mouse brain. I find NS cells can survive for at least 6 weeks after transplantation, although their migration appears limited. In adult brain, mouse NS cells differentiate into both astrocytes and morphological neurons expressing interneuron markers including Calretinin and Somatostatin. However, injected cells largely generate astrocyte in neonatal brain. These observations demonstrate that NS cells can be used as donor cells for transplantation studies. Future studies are required to evaluate how human and rat NS cell will behave after transplantation. It would also be informative to investigate whether cultured NS cells may contribute to functional repair in disease models.

Chapter 1

Introduction

The central nervous system (CNS) is the most complex system of a human body. It consists of brain and spinal cord, playing a fundamental role in controlling behaviours in the body. At the cellular level, approximately 90% of the cells of the CNS are glial cells, which physically and metabolically support the neurons that transmit electrical signals. Neurons and glia cells in the CNS both originate from a small number of stem cells or precursor cells in the developing embryo. In fact, all organs of the body originate from a population of pluripotent stem cells termed embryonic stem (ES) cells (see below). During the development, when the majority of stem cells differentiate into other cell types, some immature stem cells are maintained in certain organs such as blood and skin. These stem cells contribute to the self-repair and regeneration in some organs following trauma or diseases. Unlike the self-renewing blood or skin, the adult CNS display limited capacity of self-repair. Therefore, a long-standing dogma held that the adult brain and spinal cord were post-mitotic organs that were unable to regenerate nerve cells. It was

postulated that nervous system plasticity was achieved by "strengthening synapses" without adding new neurons Hebb (1949). The theoretical understanding of plastic processes such as learning, memory, mood, and other features of adult behavior is entrenched in this concept of a fixed number of neurons in the adult brain. As a result, research on brain plasticity has long focused on alterations in neurotransmitter receptors, numbers of synapses, structure of synapses, and transmitter release mechanisms. However, this view was gradually changed over last 40 years, when the generation of new neurons was described in adult vertebrates CNS including human (Altman, 1963; Kirsche, 1967; Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984; Burd and Nottebohm, 1985; Paton et al., 1985; Corotto et al., 1993; Lois and Alvarez-Buylla, 1994; Cameron and McKay, 1998; Eriksson et al., 1998; Kornack and Rakic, 1999, 2001; van Praag et al., 2002) (see 1.2.2 for more details).

The discovery of adult neurogenesis raises the assumption that neurogenic stem cells may exist not only in the developing embryo but also in the adult CNS (Gage, 2000; Taupin and Gage, 2002). In 1992, Reynolds and Weiss reported the first isolation and expansion of *in vitro* neural precursor cells derived from adult mouse brain (Reynolds and Weiss, 1992). Since then, cultured neural stem/precursor cells have attracted increasing interest. On one hand, *in vitro* neural stem/precursor cells offer an accessible model to investigate neurodevelopment and cell biology, as well as a promising tool for disease modelling and drug screening. On the other hand, the scaleable production of neurons from *in vitro* stem cell lines could be applied in regenerative medicine to treat nervous system disorders. During the last few years, neural stem/precursor cell cultures have been derived from various sources. In this chapter, I will introduce some concepts that are commonly used in neural stem cell research and are examined in this thesis. In addition, I will review recent studies regarding to the origin and identity of neural stem cells, as well as current strategies to expand them *in vitro*.

1.1 Definition of stem cells

1.1.1 Stem Cell and embryonic stem cell

The rigorous definition of a stem cell requires a cell possessing two properties: (1) the ability to produce unaltered daughter cells and (2) to generate progeny with different, more restricted properties (Smith, 2006). Stem cells that are able to form all cell lineages of the body are termed pluripotent stem cells. Embryonic stem (ES) cell is a type of pluripotent stem cells. They can be isolated from the inner cell mass of blastocysts and propagated indefinitely *in vitro* (Evans and Kaufman, 1981; Martin, 1981; Ying et al., 2003). ES cell will differentiate into cells of different lineages upon exposure to appropriate conditions. The neural commitment of ES cells has been demonstrated in culture conditions (Bain et al., 1995; Wiles and Johansson, 1999; Tropepe et al., 2001; Ying et al., 2003).

1.1.2 Tissue stem cell and neural stem cell

Unlike pluripotent ES cells, tissue stem cells have more limited differentiation potential. Tissue stem cells refer to stem cells that reside in, or can be derived from, a specific tissue and are able to generate cell lineages within that tissue (Smith, 2006). Neural stem cell is a type of tissue stem cell. A neural stem cell should be able to give rise to the three major cell types of the nervous system – neurons, astrocytes, and oligodendrocytes.

1.1.3 Neural progenitor/precursor cell

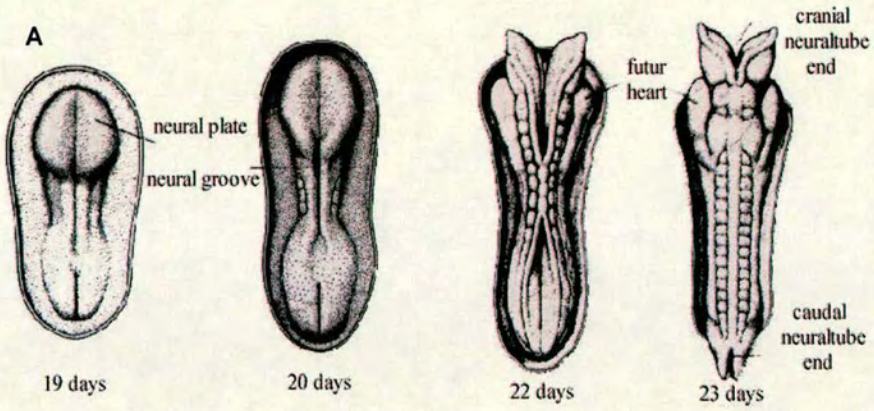
Progenitor/precursor cell is a generic terms for any dividing cell with the capacity to differentiate (Smith, 2006). In this thesis, "neural progenitor cells" and "neural precursor cells" refer to putative neural stem cells in which self-renewal has not yet been demonstrated or to those committed cells with limited self-renewal and/or differentiation capacity.

1.2 Neural stem cell *in vivo*

1.2.1 Neural development

In vertebrates, the development of the CNS occurs following gastrulation, an early phase in the embryonic development by which time all three germ layers (ectoderm, mesoderm, and endoderm) are established. The ectoderm germ layer forms a variety of body structures including the nervous system. After gastrulation, the notochord - a flexible, rod-shaped body that runs along the antero-posterior axis - is generated from mesoderm. The notochord sends signals to the overlying ectoderm and induces it to form the thick and flat neural plate. Initially, the neural plate consists of a single layer of rapidly dividing neuroepithelial cells. It is believed that all neural cells of the mature CNS are descended, either directly or indirectly, from these neuroepithelial cells. Neuroepithelial cells first display high-columnar morphology but later move laterally and away from the central axis and change into a truncated-pyramid shape. As a result, the neural plate forms the medial hinge point that later leads the neural plate to fold. The neural folds will eventually meet and fuse at the midline to form the neural tube (Fig. 1.1). The neural tube is continuous and contains primitive cerebrospinal fluids. The cephalic end of the neural tube swells to form the foetal brain and the remainder will become the spinal cord. Enlargements of the central cavity (neural tube lumen) in the region of the brain become the two lateral, third, and forth ventricles of the fully developed brain (Ladher and Schoenwolf, 2005).

The molecular mechanisms of embryonic neural induction have been studied extensively. One widely accepted theory states the abrogation of Bone morphogenetic protein (BMP) signals is central for ectodermal cells to acquire neural fates. In this model, the inhibition of BMP signaling can be modulated at multiple levels. For example, notochord secretes BMP antagonists including noggin, chordin, cerberus, and follistatin that bind and inhibit BMP ligands from engaging BMP receptors (Weinstein and Hemmati-Brivanlou, 1999;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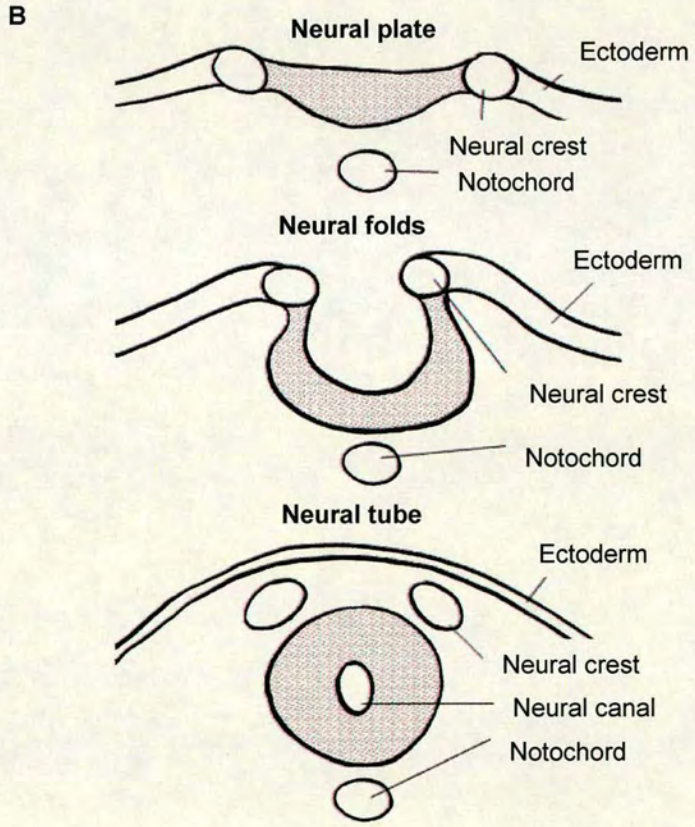
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Neural plate
Ectoderm
Neural crest
Notochord
Neural folds
Ectoderm
Neural crest
Notochord
Neural tube
Ectoderm
Neural crest
Neural canal
Notochord

Figure 1.1 Neural tube formation in human embryo.

(A) Dorsal views of primary neurulation in human embryo. The nervous system develops when the notochord induces its overlying ectoderm to become neuroectoderm and to develop into the neural plate. The neural plate folds along its central axis to form a neural groove lined on each side by a neural fold. The two neural folds fuse together and pinch off to become the neural tube. Fusion of the neural folds begins in the middle of the embryo and moves cranially and caudally. The cranial open end of the tube is the anterior (rostral) neuropore, and the caudal open end of the tube is the posterior (caudal) neuropore.

(B) Stages of neural tube closure in a transverse section. The neural plate elevates to form neural folds, which progressively appose each other, ultimately fusing to create a closed neural tube.

Modified from image at http://www.anencephalie-info.org/medical.htm

Harland, 2000; Munoz-Sanjuan and Brivanlou, 2002). In the cytosol, the inhibitory Smad6 competes with Smad4 for binding to activated Smad1, whereas Smad7 is thought to exert part of its inhibitory activities by preventing the activation of Smad1 and -2 (Nakao et al., 1997; Hata et al., 1998). Further regulation of BMP signaling can also be achieved through transcriptional regulation of BMP gene expression, which has been shown in various species (Weinstein and Hemmati-Brivanlou, 1999; Harland, 2000; Munoz-Sanjuan and Brivanlou, 2002). In addition to the default model, recent studies have suggested that some other signaling cascades are also involved in the neural induction. For example, fibroblast growth factors (FGFs) have been proposed to promote neural fates independently of BMP inhibition, but the exact mechanism is not yet clear (Wilson et al., 2001).

During early neural cell determination in the developing embryo, several transcription factors are expressed. Sox genes, such as Sox1 and Sox2, are among the earliest expressed genes in the neural plate, and appear to have a conserved function in neural cell determination across species (Sasai, 2001). Transcription factors including Otx2, Pax3, Pax7, Msx1, and Msx2 are also expressed in the neural plate (Liem et al., 1995; Ericson et al., 1996; Muhr et al., 1999). Additional neural progenitor markers such as the intermediate filament protein Nestin and the RNA-binding protein Musashi (encoded by the Msi1 and Msi2 genes) appear after Sox proteins. The expression of these two markers persists during embryo development and in some areas of the adult brain (see below). However, one needs to notice that the majority of the above markers are not stem cell specific. For example, Nestin and Musashi are expressed not only by neural stem/precursor cells, but also by more committed progenitors (Mayer-Proschel et al., 1997; Rao et al., 1998; Kaneko et al., 2000). Researchers have spent years to identify stem cell-specific markers, but little success has been achieved.

1.2.2 Neurogenesis in adult brain

As mentioned in the beginning of this thesis, due to the limited capacity for self-repair, early neuroanatomists (including Santiago Ramon y Cajal, one of the founders of modern neuroscience) considered the adult CNS was entirely post-mitotic and immutably constructed. For many years afterwards, few biologists considered adult neurogenesis a possibility, but this view has been completely changed now.

The first reports of the production of new neurons in the CNS of adult vertebrates date back to the 1960s. Two pioneers in this emerging field of research were Joseph Altman and Walter Kirsche. By labelling mitotic cells through incorporation of tritiated thymidine into newly synthesized DNA, Altman was one of the first who provided direct autoradiographic evidence for the generation of new neurons in the adult mammalian brain (Altman, 1963; Altman and Das, 1965; Altman, 1969). Kirsche employed both traditional histological methods and [3H]thymidine autoradiography to trace the origin of such neurons back to specific proliferation 'matrix zones' (Kirsche, 1967). Kirsche examined postembryonic matrix zones in no less than 23 vertebrate species, including representatives of fish, amphibians, reptiles, birds, and mammals. He demonstrated that these proliferation zones share many features across species, including their location in the vicinity of ventricles. In the 1970s and 1980s, important advances were achieved through adult neurogenesis research on non-mammalian vertebrate. In 1983, Fernando Nottebohm's lab reported that adult neurogenesis occurs in the vocal control nucleus HVC of canaries (Goldman and Nottebohm, 1983). In particular, Nottebohm and associates succeeded, for the first time, in demonstrating that newly generated cells can develop into functional neurons. These new neurons are contacted by chemical synapses, make proper connections with other brain areas, and exhibit morphological and physiological characteristics indistinguishable from older neurons (Paton and Nottebohm, 1984; Burd and Nottebohm, 1985; Paton et al., 1985). These discoveries greatly stimulated research in

the area of adult neurogenesis and led to an enormous gain in our understanding of various aspects associated with this phenomenon. Yet, these major discoveries were largely ignored by the wider scientific community, which, for a long time, perceived that adult neurogenesis as a phenomenon primarily restricted to non-mammalian vertebrates and thus of little biomedical relevance. However, this situation changed dramatically in the 1990s. In 1992, Reynolds and Weiss reported the isolation of neurogenic precursor cells from the striatum of the adult mouse brain which could be stimulated to proliferate *in vitro* (Reynolds and Weiss, 1992). Together with the finding that those cells had antigenic properties of neuroepithelial stem cells, these results suggested that a population of stem cells or precursor cells survive in the adult brain. In 1994, Kirschenbaum et al., (1994) reported an *in vitro* study suggesting that the adult human forebrain also harbors such precursor cells. Finally, with the development of new protocols to label dividing cells in the CNS, has it been confirmed that the generation of new neurons occurs in the adult mammalian brain including human (Corotto et al., 1993; Luskin, 1993; Seki and Arai, 1993; Lois and Alvarez-Buylla, 1994; Kuhn et al., 1996; Eriksson et al., 1998; Kornack and Rakic, 1999; Sanai et al., 2004).

Adult neurogenesis is now believed to occur in two principle areas of the mammal CNS: the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the forebrain ventricle (Taupin and Gage, 2002). In the dentate gyrus, newly generated neuronal cells in the subgranular layer (SGL) migrate to the granule cell layer, where they project to the CA3 area of Ammon's horn (Cameron and McKay, 1998; Gage et al., 1998; Garcia-Verdugo et al., 1998). In the SVZ, neuronal cells migrate along the rostral migratory stream (Svendsen et al.) into the olfactory bulb, in which they terminally differentiate into interneurons (Corotto et al., 1993; Lois and Alvarez-Buylla, 1994; Kornack and Rakic, 2001; Alvarez-Buylla and Garcia-Verdugo, 2002). The adult SVZ consists of various types of cells, which are divided into four groups according to their

ultrastructural characteristics (Doetsch et al., 1997): young neurons (Type A cells), glia cells displaying astrocytic characteristics (Type B cells), highly proliferative cells (Type C cells), and ependymal cells (Type E cells). It is believed that astrocyte like Type B cells generate transient amplifying C cells, which later produce immature neuronal A cells that will finally migrate into the olfactory bulb and differentiate into mature interneurons (see blow for details) (Doetsch et al., 1999; Alvarez-Buylla and Garcia-Verdugo, 2002).

1.3 The identity of neural stem cell

Due to a lack of specific markers, the identification of neural stem cells *in vivo* is not easy. In the developing embryo, a cell population called radial glia are suggested to be neural stem cells. Radial glia cells were originally named epithelial cells, spongioblasts, radial cells, or fetal ependymal cells. They appear in neuroepithelial tissue concurrent with the onset of neurogenesis (see above). Radial glia divide in the ventricular zone of the foetal neocortex and have a long basal process extending from their cell body through the parenchyma toward the brain surface. They exhibit dynamic interkinetic nuclear migration, during which the nuclei oscillate between the ventricular and cortical surfaces (Alvarez-Buylla et al., 1998; Noctor et al., 2001). Radial glia and neuroepithelial cells share many characteristics. They exhibit similar division patterns, apical-basal polarity, and the expression of immature neural markers such as nestin (Alvarez-Buylla et al., 2001). Therefore, it is suggested radial glial cells are transformed directly from neuroepithelial cells (Malatesta et al., 2003; Gotz and Huttner, 2005; Ihrie and Alvarez-Buylla, 2007). Radial glia cells have anatomical features of astroglial cells, such as endfeet on blood vessels, intermediate filaments, and glycogen granules. In addition, radial glia also express a group of glia markers including RC2, BLBP, GLAST, and in primates, GFAP (Bentivoglio and Mazzarello, 1999; Rakic, 2003). For many years, radial glia cells were classically viewed as immature glial cells that guide neuronal migration and function as scaffolding for brain development. However, this view has been changed

recently since studies in songbirds (Alvarez-Buylla et al., 1990), where radial glia persist into adult life. Work in the developing rodent brain indicate that radial glia also function as neural progenitors that give rise to neurons and glia (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001; Seri et al., 2001; Malatesta et al., 2003). Although it is not yet clear whether a given radial glia cell can generate the multitude of cell types in brain, some researchers suggest that radial glia are, if not the only, at least the primary progenitors of the embryonic forebrain (Anthony et al., 2004).

In the adult brain, there are three theories with regard to the identity of neurogenic cell populations. The first theory states that newly generated neurons originate from a population of GFAP expressing cells in the SVZ and SGL. Doetsch et al. (1999) and Seri et al. (2001) demonstrated that SVZ and SGL astrocytes divided to generate immature precursors and neuroblasts after immature precursor cells were removed by antimitotic treatment. Moreover, Doetsch et al. showed SVZ astrocytes were capable of generating multipotent neurospheres *in vitro,* providing addition evidence for the neurogenic potential of SVZ GFAP expressing cells (Doetsch et al. 1999). Garcia et al. (2004) developed two strategies to determine the relative contribution of GFAP-expressing progenitor cells to constitutive neurogenesis in the adult brain. One strategy combined the targeted expression of herpes simplex virus thymidine kinase (HSV-TK) with delivery of the antiviral agent ganciclovir (GCV) to achieve the specific and inducible ablation of dividing GFAP-expressing cells *in vivo*. Ablation of dividing GFAP expressing cells in the adult mouse SVZ and SGL stopped the generation of new neuronal cells in the olfactory bulb and the hippocampal dentate gyrus, an observation which was also reported by Morshead et al., (2003). The other strategy allowed fate mapping of progeny cells derived from GFAP-expressing cells by using the targeted expression of Cre recombinase (Cre) to excise a loxP-flanked stop signal and activate reporter gene expression from an independent ubiquitous promoter. The fate mapping showed that essentially all new

neuronal cells generated in the adult mouse forebrain are derived from progenitor cells expressed GFAP (Garcia et al., 2004). The second theory contends that newly generated neuronal cells originate from a population of ependymal cells in the SVZ. In 1999, Johansson et al. reported that ciliated ependymal cells displayed proliferative and multiple differentiation potentials *in vitro*, and that DiI-labelled ependymal cells appear as a source for olfactory bulb neurons (Johansson et al., 1999). However, this theory remains to be controversial, as other researchers show contradictory evidence challenging the initial report (Doetsch et al. 1999; Chiasson et al., 1999; Capela and Temple 2002). The third theory identifies adult neural stem cells as a population of GFAP negative cells. Two independent studies based on flow cytometry showed no expression or only partial expression of GFAP in adult neural precursor cells derived from the SVZ area. In one study, neural precursor cells were isolated and characterised by negative selection using lectin peanut agglutinin (Rietze et al., 2001). In the second study, neural progenitor cells were isolated using the carbohydrate moiety Lewis X, also known as CD15 or SSEA-1 (Capela and Temple, 2002). Although these findings raise the possibility that a population of adult neural stem cells may not express GFAP, one cannot draw any meaningful conclusion before the relationship between cultured neural stem cells and neural precursor cells *in vivo* are carefully analyzed. Among the three theories, the glial-origin for neural stem cells in the adult brain has received much support. and recent studies have extended this theory by showing that the adult neurogenic astrocytes may originate from neonatal radial glia (Tramontin et al., 2003; Merkle et al., 2004). Together with the studies of radial glia, a model emerges that the population of neural stem cells *in vivo* develops from embryonic neuroepithelial cells to radial glia cells and then to adult neurogenic astrocytes (Merkle, et al., 2004).

1.4 Regulation of neural stem cells

Although it is now well accepted that new neurons are generated throughout life, the

mechanisms of how a pool of neural precursor cells is maintained and how these cells are induced to manifest multi-lineage potency remain elusive. *In vivo* and *in vitro* studies during the last few years have recognized complicated and delicate networks being involved in the regulation of neural stem/precursor cell behaviour. Generally, the regulating mechanisms can be divided into three categories, the intrinsic mechanisms (transcription factors and epigenetic modifications of proteins and DNA), extrinsic mechanisms (extracellular factors and/or substrates as well as their intracellular signalling machinery), and signalling pathways (e.g. Wnt, BMP, Shh, Notch, JAK-STAT signalling etc.). These regulatory machineries act either individually or in combination during embryonic development and adulthood, being concerned with fate decision of not only neural stem cells but also precursors of other organs. Adding to the complexity, increasing evidence have show that signalling pathways cross-talk extensively, and that the same signal may promote different fates depending on the cellular context (Guillemot, 2007). In the following sections, I will review EGF, FGF, BMP, and Wnt signals that are involved in the research described in the later chapters.

1.4.1 EGF and FGF signals

FGFs constitute a large family of structurally related polypeptides. There are at least 23 members of the FGF family, of which 10 are expressed in the CNS (Ford-Perriss et al., 2001; Dono, 2003). The FGFs are involved in a variety of biological mechanisms, and their ligands and receptors are expressed by neural precursor cells from the earliest phases of CNS development (Ford-Perriss et al., 2001). It has been shown that FGF signals contribute to the regulation of proliferation, migration, survival of neurons and glial, patterning of CNS regions, cerebellar development, and cerebral cortex size (Ye et al., 1998; Fukuchi-Shimogori and Grove, 2001). FGF2 is the member of the FGF family that has been most extensively studied. FGF2 is essential for normal neurogenesis and has been found in the forebrain as early as embryonic day 9 (Powell et al., 1991; Weise et al.,

1993). Mice lacking FGF2 exhibit decreased numbers of neurons and glia, whereas injection of the ligand into the embryonic SVZ produces the opposite effect (Craig et al., 1996; Kuhn et al., 1997; Vaccarino et al., 1999). It has been shown that FGF2 increases the rounds of cell division, but it has no effect on the length of the cell cycle (Vaccarino et al., 1999). Mice with deficient FGF2 also show abnormalities in the laminar organization of the cerebral cortex and defects in the hippocampus and spinal cord (Dono et al., 1998; Ortega et al., 1998).

The pro-proliferation effects of FGF2 can be duplicated by another mitogen EGF both *in vivo* and *in vitro* (Reynolds and Weiss, 1992; Morshead et al., 1994; Craig et al., 1996; Kuhn et al., 1997). However, the biological functions of EGF and FGF2 are not identical. Kuhn et al. (1997) showed that intracerebroventricular infusion of FGF2 not only increased the number of newborn cells in the SVZ but also enhanced the generation of new neurons in the olfactory bulb. However, infusion of EGF reduced the total number of newborn neurons reaching the olfactory bulb and substantially enhanced the generation of astrocytes (Kuhn et al., 1997).

Since it appears that EGF and FGFs are key regulators of CNS development, it is likely that mutation of EGF/FGFs or their receptors are involved in several human CNS disorders. Generation of tissue-specific FGF for FGF receptor knockouts will probably help to dissect the function of EGF/FGFs in the CNS in more detail.

1.4.2 BMPs and BMP antagonists

In addition to EGF and FGF2, BMP signals have recently been identified to play important roles in neural stem cell fate decision. BMPs have been shown to promote neuronal differentiation in early cortical explant cultures (E12-13 in the mouse) (Li et al., 1998), but enhance astrocyte differentiation in older progenitor cultures (Gross et al.,

1996). In the adult SVZ niche. Lim et al (2000) showed that SVZ neural precursor cells expressed BMPs and BMP receptors. *In vivo* and *in vitro* observations indicate that BMP signals inhibit SVZ type B and type C generate new neurons, but such inhibitory effects are antagonized by Noggin produced by ependymal cells (Lim et al., 2000). Although it was not shown whether Noggin promote the SVZ cell proliferation *in vivo*, BMP and BMP antagonist signals may act as necessary components in the regulation of adult neural progenitor behaviour.

BMPs are a group of structurally and functionally related proteins belonging to the transforming growth factor (TGF) super family. In addition to BMPs, this family also includes the TGFβs, growth and differentiation factors (GDFs), Activins, Inhibins, Leftys, Nodal, and Mullerian inhibitory substance. BMPs were originally identified as regulators of cartilage and bone formation, but now they have been recognized to play important roles in embryogenesis and morphogenesis in various tissues and organs (Ebendal et al., 1998; Kawabata et al., 1998). BMP signals display various effects on different cell types. In mouse ES cells, BMPs act in combination with LIF (LIF/STAT3 signaling) to sustain pluripotency by inducing Inhibitor of differentiation (Id) genes expression (Ying et al., 2003). In the absence of LIF, the activation of BMP signals lead to differentiation of ES into non-neural lineages (Ying et al., 2003). In neural stem/precursor cells, BMP signaling generally exhibits an antiproliferative and astrocyte differentiation-inducing effect. It is indicated that BMPs enhance the expression of Id proteins, which sequester E proteins, the dimerization partners of proneural bHLH transcription factors such as Neurogenin1 and Mash1, thereby blocking their activity and promoting the degradation of Mash1 (Nakashima et al., 2001; Vinals et al. 2004; Guillemot, 2007). In addition, BMPs may also inhibit oligodendrogenesis by activation of Id proteins that dimerize with Olig proteins and their E protein partners, and thus block oligodendroglial promoting activity (Samanta and Kessler, 2004)

BMPs exert their effects by binding to their cognate receptors. Extracellular BMPs bind to type I receptors and induce the formation of a heteromeric complex with type II receptors. Once the heteromeric complex is formed, type II receptors phosphorylate type I receptor by the constitutively active serine/threonine kinase. In the 'canonical' BMP signaling pathway (also known as the BMP-Smad pathway), type II receptors activate receptor-mediated Smads (R-Smads), Smad 1/5/8. This activation enables the R-Smad to complex with the common mediator Smad (Co-Smad), Smad4, and the R-Smad/Smad4 complex enters the nucleus to activate or repress target genes depending on which nuclear cofactors are present. Smad6 and Smad7 comprise the third class of Smads, the inhibitory Smads. The expression of Smad6 and Smad7 are induced by BMP signaling, establishing negative feedback loops (Fig. 1.2) (Kawabata et al., 1998). In addition to the "canonical" signaling pathway, recent studies suggest that a mitogen-activated protein kinase (MAPK) pathway may mediate BMP signal transduction, which is known as BMP-MAPK pathway. In this pathway, BMP signals can be transduced by TGF-b activated kinase 1 (TAK1), a MAP kinase kinase kinase (MAPKKK), and TAK1 binding protein 1 (TAB1) that has been implicated in the activation of the JNK and p38 MAPK signaling pathways (Fig. 1.2) (Yamaguchi et al., 1995; Shibuya et al., 1998; von Bubnoff and Cho, 2001).

BMP signaling has crosstalk with other signal transduction pathways, for example the Erk–MAPK, the Wnt/Ca2+, the TGF-b/activin, and the JAK–STAT pathway (von Bubnoff and Cho, 2001; Attisano and Wrana, 2002; Derynck and Zhang, 2003). In the Erk-MAPK pathway, ligands such as EGF/FGF activate receptor tyrosine kinases (RTKs) that subsequently activate the extracellular signal regulated kinase (Merkle et al., 2004). It has been revealed that Erk kinases phosphorylate serine within the specific region linking the inhibitory and effector domains of Smad1, and therefore inhibit Smad1 nuclear accumulation (Kretzschmar et al., 1997). This mechanism may underlie the observed

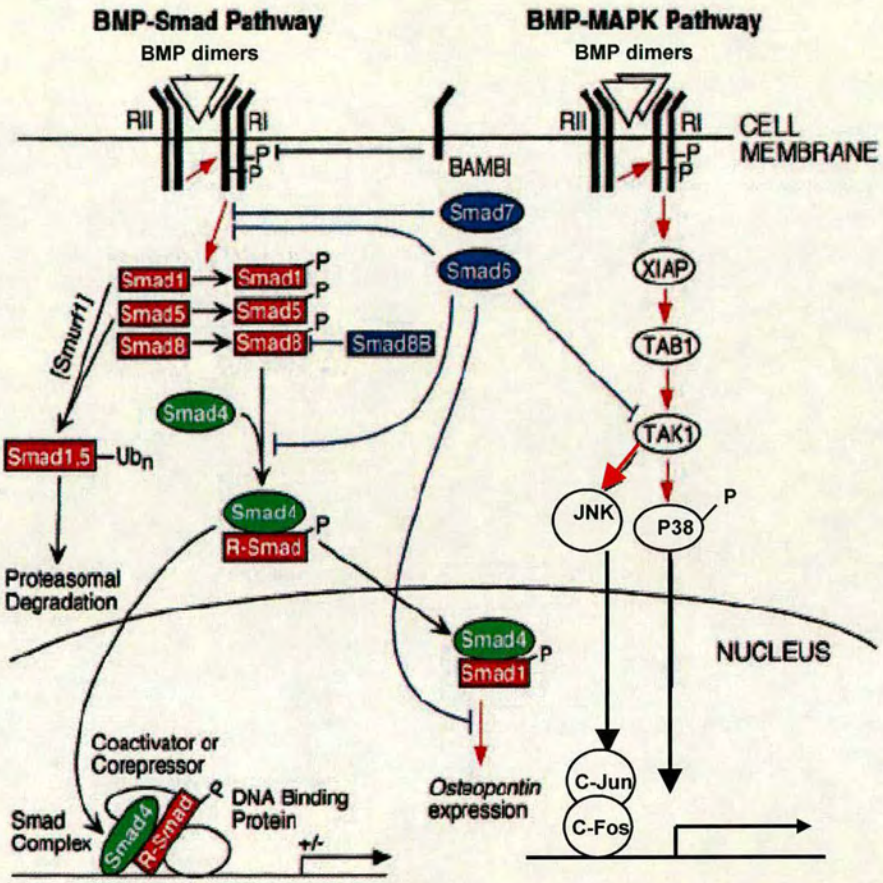

BMP-Smad Pathway
BMP dimers
BMP-MAPK Pathway
BMP dimers
RII
RI
BAMBI
CELL MEMBRANE
Smad7
Smad6
Smad1
Smad5
Smad8
Smad8B
[Smurf1]
Smad1,5
Ubn
Smad4
R-Smad
Proteasomal Degradation
XIAP
TAB1
TAK1
JNK
P38
NUCLEUS
Coactivator or Corepressor
DNA Binding Protein
Smad Complex
+/-
Osteopontin expression
C-Jun
C-Fos

Figure 1.2 The canonical BMP–Smad pathway and the BMP–MAPK pathway.

In the BMP–Smad pathway, BMPs dimers bind to the receptor complex, leading to phosphorylation of the type I receptor (RI) by the type II receptor (RII), which in turn phosphorylates an appropriate R-Smad (Smad1, -5, or -8). This phosphorylation enables the R-Smad to complex with the Co-Smad, Smad4, and the R-Smad/Smad4 complex enters the nucleus to activate or repress target genes depending on which nuclear cofactors are present. Smad6, Smad7, Smad8B, BAMBI, and Smurf1 all inhibit the BMP–Smad pathway at different levels. Smad6, Smad7, and BAMBI are induced by BMP signaling, establishing negative feedback loops. In the BMP–MAPK pathway, activated BMP receptors may interact with XIAP, which in turn activates the MAPKKK TAK1 by interacting with TAB1. Smad6 inhibits the BMP–MAPK pathway as well. Red indicates activation and blue indicates inhibition. Smads that are part of or regulate the BMP-Smad pathway are colored: Smad1, -5, and -8 are red, the Co-Smad Smad4 is green, and the I-Smads and Smad8B are blue.

Modified from Von Bubnoff and Cho, Dev Bio, 2001

opposing effects of mitogenic factors and BMPs during vertebrate development. For example, EGF can oppose the BMP2-dependent induction of osteogenic differentiation, and FGF opposes BMP4's ability to induce interdigital apoptosis during digit formation (Niswander and Martin, 1993; Ganan et al., 1996; Neubuser et al., 1997; Lillien and Raphael, 2000).

A group of molecules, including Noggin and Chordin, have been identified as BMP antagonists. Noggin and Chordin belong to a class of polypeptides that bind to BMPs and consequently prevent their activation of BMP receptors. Noggin and Chordin have been detected in many foetal and adult tissues such as brain, liver, lung, and skeletal muscle, suggesting they have multiple roles (Smith and Harland, 1992; Valenzuela et al., 1995; Brunet et al., 1998; Larrain et al., 2000; Coffinier et al., 2001). In addition to Noggin and Chordin, members of cerberus/DAN family have been identified as BMP antagonists as well. Mammalian DAN family members include DAN (DAND1), Gremlin/Drm (DAND2), PRDC (Protein Related to Dan and Cerberus; DAND3), Cerberus (DAND4), and COCO (DAND5). The expression of Gremlin and PRDC has been detected in both brain and spinal cord, but their functions are not clear.

1.4.3 Wnt signaling

The Wnt family consists of a group of cysteine-rich, secreted glycoproteins. The name Wnt was coined as a combination of Wg (wingless) and Int. The Wg gene was identified as a locus in Drosophila required in each segment for the establishment of normal pattern (Nusslein-Volhard and Wieschaus, 1980; Wu and Cohen, 2002). The int loci (there were 2 termed int-1 and int-2) were originally identified in mice as sights of frequent integration (hence int loci) of the Moloney murine leukemia virus (Nusse et al., 1984). It was shown that the murine int-1 locus was homologous to Drosophila Wg and the family name was changed to Wnt to reflect both origins. There are at least 18 mammalian members of the

Wnt family, which have been shown to play important roles in embryonic patterning through the regulation of cell proliferation, fate decisions, tissue polarity, and cell movements (Nelson and Nusse, 2004; Ciani and Salinas, 2005; Gordon and Nusse, 2006).

In the CNS development, the activities of Wnt signals are complex. At early stages of cortical development, Wnt signals have been shown to promote progenitor proliferation. Overexpression of a stabilized form of β-catenin (see below) results in overproliferation of cortical progenitors (Chenn and Walsh, 2003; Zechner et al., 2003). At later developmental stages (after E13.5 in the mouse), Wnt signals shift their roles to promote neurogenesis (Hirabayashi et al., 2004). In the adult CNS, Wnts also have essential functions, as aberrant signalling by Wnt pathways is linked to impaired neurogenesis in hippocampus and a range of diseases including cancer (Moon et al., 2004; Zhou et al., 2004; Reya and Clevers, 2005; Lie et al., 2005).

The Wnt pathways are remarkably conserved in a wide variety of organisms and involve a large number of proteins that can regulate the production of Wnt signaling molecules, their interactions with receptors, and the responses of target cells. The canonical Wnt pathway is known as the "Wnt-β-catenin" signaling. In this pathway, Wnt proteins bind to seven-transmembrane receptors Frizzleds (Fzd) to activate Dishevelled (DSH) family proteins. Activated DSH is a key component of a membrane-associated Wnt receptor complex that inhibits the formation of a second protein complex that includes axin, GSK-3, and APC. The axin/GSK-3/APC complex normally promotes the proteolytic degradation of the β-catenin intracellular signaling molecule. When it is inhibited, a pool of cytoplasmic β-catenin stabilizes, and some β-catenin is able to translocate into the nucleus, where it interacts with regulators such as T-cell factor/lymphocyte enhancer factor (Tcf/Lef) transcription factors to regulate expression of target genes (Nelson and Nusse, 2004; Reya and Clevers, 2005). In addition to the canonical Wnt pathway, much

work has been done recently on non-canonical Wnt pathways, known as the JNK/AP1 dependent Wnt/PCP pathway and the PKC/CAMKII/NFAT dependent Wnt/Ca2+ pathway. The non-canonical pathways differ from the β-catenin pathway in their dependency on the type of G-proteins that they require for activation (Strutt, 2003; Veeman et al., 2003; Widelitz, 2005).

1.4.4 Other regulatory machineries

As mentioned earlier, many other growth factors are involved in regulating neural stem cell self-renewal and differentiation. For example, PDGF has been extensively characterized for its role in the proliferation and survival of oligodendrocyte precursors (Barres and Raff, 1994; Fruttiger et al., 1999). Moreover, PDGF is also identified to promote the survival and proliferation of progenitors as well as neuronal differentiation when added to cortical progenitor cultures (Johe et al., 1996; Williams et al., 1997). These effects, however, are mediated by distinct pathways, possibly involving downstream cascades of tyrosine kinase receptor and the PI3 kinase-Akt pathway (Barnabe-Heider and Miller, 2003). Interestingly, it has been discovered recently that PDGF receptor is expressed by a subset of stem cells in the adult SVZ, and that PDGF may be involved in a fate choice between oligodendroglial and neuronal lineages in stem cells of the adult SVZ (Jackson et al., 2006).

The role of Notch pathway has been well-established in maintaining progenitors in an undifferentiated state during the neurogenic phase of CNS development (Artavanis-Tsakonas et al., 1999; Mizutani and Saito, 2005). Many of the Notch activities of the Notch pathway are mediated by the Hes transcriptional repressors, which includes Hes1 and Hes5 in the CNS. Hes1 and Hes5 are essential for keeping neuroepithelial cells or radial glial cells at an undifferentiated state (Hatakeyama et al., 2004; Nakamura et al., 2000; Ohtsuka et al., 2001), but they also have been implicated in the generation of Muller

glial cells in the retina (Hojo et al., 2000). Recently, investigations have show that the Notch signaling has a more complex role in neural development, including promoting the transformation of neuroepithelial progenitors into radial glial cells (Lowell et al., 2006) and later on the differentiation of radial glial cells into astrocytes (Anthony et al., 2005; Gotz and Huttner, 2005). It is speculated that the pro-astroglial effects are possibly through repressing of proneural genes such as Ngn1, Ngn2 and Mash1 (Guillemot, 2007) or activating the transcription factor STAT3, a potent inducer of astrogliogenesis, through recruitment of JAK2 kinase (Kamakura et al., 2004).

In early neural development, Shh expressed by cells of the notochord and floor plate and is responsible for specifying the subtype identity of neurons generated in the ventral half of the neural tube (Placzek, 1995). Shh acts as a graded signal inducing diverse neuronal subtypes at different concentration thresholds, and its activity is detectable in regions that can be several cell diameters from its sources (Marigo and Tabin, 1996; Ericson et al., 1996). It has been shown that Shh is necessary and sufficient to induce the differentiation of most ventral neuronal subtypes either *in vivo* or *in vitro* (Placzek, 1995; Marti et al., 1995; Roelink et al., 1995; Ericson et al., 1996). In addition to determining diverse neuronal fates, Shh signals also promotes the generation of ventrally-born oligodendrocyte precursor cells and subsequent oligodendrogenesis by inducing Olig1 and Olig2 expression, two essential determinants of the oligodendrocyte fate (Lu et al., 2000; Alberta et al., 2001; Nery et al., 2001; Tekki-Kessaris et al., 2001; Yung et al., 2002)

In addition to the regulating machineries mentioned above, several groups of transcriptional factors, which are not reviewed in details in this thesis, have also been extensively studied for their roles in selecting cell fates of neural precursor cells. Generally, these transcription factors can be catalogued into genes promoting neurogenesis (e.g. Mash1, Neurogenin1, Neurogenin2, and Pax6) or genes favouring glial

fates (e.g. Olig genes, Nkx2 genes, and Sox E genes). Yet, it is worth a mention that some transcription factors, such as Mash1, have been implicated in multiple roles, not only in neurogenesis but also in oligodendrogenesis.

1.5 Neural stem cell *in vitro*

1.5.1 Source of in vitro neural stem cells

To investigate the existence of neural stem cells *in vivo* and also to evaluate the actions of growth factors, investigators have aimed at isolating and characterizing cells with self-renewing and multipotent properties from different sources. As mammalian neural stem cells largely present in developing CNS, it might seem obvious that foetal neural tissue would be an important source from which these cells could be isolated. However, there is also evidence that neural precursor cells with multi-potential can be generated from adult tissues or tumor samples within the cell culture environment. Also, the ability to differentiate embryonic stem cells to neural lineages provides an alternative way to generate neural stem cells in unlimited numbers *in vitro*. Here, I will discuss each of these potential sources below.

Neural precursor cells can be obtained from dissociation of foetal CNS tissues. However, a major hurdle to subsequent in vitro isolation and characterization of these cells is the heterogeneity of primary cultures. Although the development of cell type specific monoclonal antibodies such as Nestin (Rat 401) allows identification of possible stem cells (Hockfield and McKay, 1985; Lendahl et al., 1990), to date no markers have been found specific for neural stem cells. In addition, most identified markers are intracellular, which limits their use for stem cell enrichment unless transgenic mice with reporter gene expression are generated (Roy et al., 2000; Kawaguchi et al., 2001). Recently, some cell surface markers have been identified that can be applied to enrich neural precursor cells *in vitor*. These cell surface markers include CD15 (also called SSEA1/LeX), CD133 (also

known as (Prominin) (Capela and Temple, 2006; Uchida et al., 2000), and more recently reported integrins (Hall et al., 2006). Identification of additional cell surface markers that can be used individually or in combination should enable isolation of neural stem cells or precursors more specifically.

The use of cell lines to model events of normal development became widespread in the mid-1970s when the first tumor derived neural cell lines were derived. Among these were those derived from neuroblastomas, glioblastomas and the rat pheochromocytoma PC12 (reviewed in: (Gottlieb, 2002). PC12 cells and rat C6 glioma line have been proven invaluable to model processes such as neurotransmitter release and signalling pathways (Kondo et al., 2004). However, the limitation of tumor-derived cell lines is that they contain significant genetic and epigenetic alterations. As an alternative way, a number of laboratories in the 1980s developed strategies that exploit oncogenes to promote continuous cell division among non-tumor cells (reviewed in: (Gottlieb, 2002). Rodent and human cell lines produced by this approach show phenotypes closely resembling those of normal immature neural cells (Cattaneo and Conti, 1998). In addition to providing continuously propagating cell populations, the advantages of perpetuated cell lines are that the region and developmental stage of the target population of stem cells can be chosen at will, and that reversible oncogenes can be utilized to allow the replicative signal to be turned off. Yet, since the transforming oncogenes are randomly inserted into the genome, transgenes are expressed in patterns strongly influenced by the site of insertion. As a result, two cell lines with insertion of the identical oncogene at different chromosomal sites could have different phenotypes (Gottlieb, 2002).

ES cells are totipotent cells derived from the early embryo. Mouse ES cells were first isolated in 1981 by Evans & Kaufman (1981) and Martin (1981). It is now known that Mouse ES cells can be maintained in vitro in the absence of differentiation through a

combination of cell extrinsic signals (LIF/gp130 and BMP), and cell intrinsic determinants, such as the transcription factors Sox2, Oct4 and Nanog (Chambers and Smith, 2004). For differentiation of ES cells into neural lineages, initial reports showed that neurons can be efficiently generated through the exposure of ES cells to retinoic acid and serum following LIF withdrawal (Bain et al., 1995; Strubing et al., 1995; Zupanc and Clint, 2003). Modifications to this protocol were developed showing neural precursors could be induced and enriched in a serum free basal media without exposure to retinoic acid (Okabe et al., 1996). Some other researchers also tried co-culture with a stromal cell line PA6 or applying conditioned media (Rathjen et al., 1999; Kawasaki et al., 2000). In 2003, our lab also reported that Sox1 expressing neuroepithelial cells can be derived from mouse ES cells under adherent culture conditions (Ying et al., 2003). Yet, a drawback of current ES cell neural differentiation protocols is that the culture contains non-neural cells as well as residual ES cells. A strategy developed to overcome this issue is known as 'lineage selection', in which a reporter or drug selectable marker is 'knocked-in' through gene targeting, or expressed as a transgene under cell-type specific promoter elements (Li et al., 2001).

With the above improved strategies, the derivation of neural precursor cells from human ES cells has been widely tested since its first derivation in 1998 (Thomson et al., 1998). Generally, the first steps to induce human ES cells under neural differentiation may involve removal of self-renewal supporting factors, forming cell aggregates, exposure to retinoic acid, inhibiting BMP signaling, and/or co-culture with stromal feeder cells, such as HepG2 or PA6. Detailed accounts and comparison of methods for neuroepithelial differentiation from human ES cells have been discussed elsewhere (Murry and Keller, 2008; Zhang et al., 2008). Yet, the biggest challenge for generation neural stem cell from human ES cells is weather an established protocol can be use repeatedly and equally efficient. This challenge was possibly raised by the fact that ES cell lines to date were

derived and maintained in very different conditions. As a result, neural differentiation efficiency and derived phenotypes vary significantly between cell lines. In addition, although some ES cell derived neural progenitors resemble adult and fetal neural progenitors in their trilineage capacity, microarray and DNA methylation assays indicate that there are many differences between these two progenitor populations (Shin et al., 2007). In this thesis, the derivation of human and rat neural stem cell lines were focused on using foetal tissue (see Chapter 2 and 3).

1.5.2 in vitro expansion of neural stem cells

To date, two major methods have been developed to propagate neural precursor or stem cells *in vitro*: the neurosphere culture and the monolayer culture. In 1992, Weiss and Reynolds reported for the first time that neural precursor cells derived from adult mouse striatum (possibly contaminating SVZ cells) could be expanded in suspension cultures without immortalization (Reynolds and Weiss, 1992). In the presence of EGF, cultured cells form aggregates termed "neurospheres", in which a small percentage of cells continue to divide and contribute to the increasing sphere size and formation of secondary spheres. When EGF is withdrawn, neurons, astrocytes, and oligodendrocytes could be found within neurospheres (Reynolds and Weiss, 1992). Since then, neurosphere culture has become the most common method to propagate neural precursor cells *in vitro*. However, this culture system has significant limitations. First, the cell populations in neurospheres are heterogeneous, which makes it hard to determine the quantity and identity of neurosphere-forming cells (Suslov et al., 2002; Reynolds and Rietze, 2005; Singec et al., 2006). Second, neurosphere cultures are not stable. They are often accompanied by progressive loss of self-renewal and differentiation capacity (Ostenfeld et al., 2000; Reynolds and Rietze, 2005). In addition, it has been shown that committed progenitors can also generate sphere-like aggregates (Seaberg and van der Kooy, 2002), and that the size, number, and variable cellular compositions of neurospheres may not

accurately reflect the proliferation activity, self-renewal capacity and developmental potential of the original sphere-forming cells (Singec et al., 2006). Thus, even if neurospheres are generated in clonal conditions, one cannot definitively infer the existence of neural stem cells in individual neurospheres (Reynolds and Rietze, 2005).

An alternative approach to grow neural precursor cells *in vitro* is monolayer culture (Ray et al. 1993). In this approach, foetal rat hippocampus is dissociated mechanically and plated on an adhesive substrate in a defined medium. In the absence of bFGF, many cells died or were unhealthy. Addition of bFGF had a survival effect on neurons and also caused precursor cells to proliferate. The same approach was later applied by the same group to produce continuously replicating cell lines from adult hippocampal progenitors (Palmer et al. 1997). However, since the karyotypes of clonal cell lines were abnormal, the identity of neural precursor cells in the above studies remains controversial. Monolayer culture conditions were also established to grow neural precursor cells from mouse ES cells. In 1996, Ron McKay's lab reported an enriched population of adherent neuroepithelial precursor cells derived from ES cells, which proliferated in the presence of basic fibroblast growth factor (bFGF) and were able to differentiate into both neurons and glia (Okabe et al., 1996). By further differentiating the cells in serum containing medium, the neurons express a wide variety of neuron-specific genes and generate both excitatory and inhibitory synaptic connections (Okabe et al., 1996). However, since this cell line was only characterized at early passages and among non-clonal populations, it is not clear whether the culture contain true neural stem cells so that the neurogenic potential may retain.

1.6 Adherent mouse NS cells

In order to establish an undifferentiated and stable neurogenic stem cell population *in vitro*, the Smith lab has recently derived adherent and clonogenic mouse neural stem cells,

which are termed NS (neural stem) cells to be distinguished from other neural progenitor cultures (Conti et al., 2005; Pollard et al., 2006). Mouse NS cell lines can be derived from various sources, including ES cells, foetal neural tissue, and adult brain (Conti et al., 2005; Pollard et al., 2006; and unpublished data). To obtain a foetal brain NS cell line, mouse cortices from mouse embryos at embryonic day 12.5-16.5 were dissected and dissociated into single cells. Dissociated cells were transiently expanded in suspension cultures to allow the formation of neurospheres, which were later re-dissociated and plated on gelatine substrates to form monolayer NS cell lines. Independent mouse NS cell lines display great similarities. They all express neural precursor/radial markers including Nestin, BLBP, RC2, 3CB2, GLAST, Vimentin, Olig2, and Sox2. Time-lapse videomicroscopy indicates mouse NS cells exhibit dynamic interkinetic nuclear migration, similar to neuroepithelial cells and radial glia. In the presence of EGF and FGF2, mouse NS cells undergo symmetrical division and retain multi-lineage differentiation capacity. Neuronal differentiation can be triggered by sequential withdrawal of, first EGF, then FGF2 for 2 weeks. Oligodendrocytes can be derived when cells are treated with PDGF, T3, and ascorbic acid (Glaser et al., 2007). Mouse NS cells generate a pure GFAP+ astrocyte population upon exposure to serum or BMP4 in the absence of EGF and FGF2. In short, mouse NS cells represent self-renewing and tripotent neural stem cell population that can be stably expanded *in vitro.*

1.7 Research aims and thesis organizations

Based on the observations with mouse NS cells, the research aims of my PhD program are: 1) to explore the possibility of establishing adherent human and rat NS cell lines (from both brain and spinal cord tissue); 2) to characterize rat and human NS cell lines after prolonged expansion; 3) to explore the possible signaling pathways that regulate the NS cell proliferation and differentiation; and 4) to investigate how NS cells survive, proliferate, or differentiate when they are transplanted into adult brain.

The research data and experimental procedures of my PhD project will be described in the following chapters. Chapter 2 reports the derivation and long-term characterization of human foetal NS cell lines. Chapter 3 examines the derivation of rat NS cells and the mechanisms of NS cell quiescence. Chapter 4 describes the preliminary transplantation experiments using green fluorescent protein (GFP) labelled mouse NS cells. In Chapter 5, I will summarize the similarities and differences between NS cells from different species, and point out the future work with NS cells. Introductions, experimental methods, and discussions relating to the above topics will be enclosed in each chapter separately. Reference and published papers are attached at the end as appendixes.

Chapter 2

Long-term tripotent differentiation capacity of human NS cells in adherent culture

2.1 Introduction

2.1.1 Human neural precursor cells in vitro

Aside form the knowledge that the human CNS arises from a highly potent cell population in the developing embryo, little is known about the nature of human neural stem/precursor cells. One of the major obstacles standing in the way of research is limited access to human neural tissue. If human neural stem cells could be cultured as *in vitro* cell lines,

they would provide an accessible model system to investigate human neurodevelopment and cell biology. In addition, cultured human neural stem cells would offer a renewable resource for neurodegenerative disease studies and would be suitable for drug screening. Lastly, scaleable production of in vitro human neurons from stem cell lines is a first step towards their use in regenerative medicine.

However, until the late 1990's, the only cell line that could consistently generate human neuronal cells *in vitro* was the teratocarcinoma derived NTERA-2 cells. This is a transformed cell line that requires complicated manipulations to induce differentiation (Andrews, 1984; Pleasure et al., 1992). These limitations led to the exploration of alternative sources and approaches to derive human neural stem cells *in vitro*. Human foetal brain and spinal cord contain proliferating neural progenitor cells, which are potential sources for deriving *in vitro* cell lines.

In 1997, Sah et al. established the first immortalized human foetal neural precursor cell line that displayed stable proliferation and bipotent differentiation potential in the presence of EGF and FGF2 (Sah et al., 1997). The immortalizing oncogene, avian *v-myc*, was retrovirally expressed and could be pharmacologically controlled due to the presence of tetracycline-regulated elements. A few years later, two independent reports additionally described oncogene immortalized neural stem cell lines named H6 and HNSC100 cell lines (Flax et al., 1998; Villa et al., 2000). Although these cell lines were generated using different starting cellular materials (H6 was derived from freshly obtained embryonic tissue while HNSC100 from established precursor cultures), they were perpetuated using the same *v-myc* coding retroviral vector. These cell lines were multipotent *in vitro*. When transplanted into the newborn mouse brain, they could also generate both neurons and glia.

In addition to oncogene perpetuated cell lines, researchers have also explored the possibility of expanding human foetal neural precursors in neurosphere cultures (Svendsen et al., 1998; Carpenter et al., 1999; Riaz et al., 2002). However, since the cell populations in neurosphere are heterogeneous (see Chapter 1), it is hard to determine the quantity and identity of neurosphere-forming cells (Suslov et al., 2002; Reynolds and Rietze, 2005; Singec et al., 2006). A few researchers have explored expansion of human neural precursor cells derived from foetal or adult tissue using adherent cultures (Palmer et al., 2001; Yan et al., 2007). Yet, these cultures still appeared to be heterogeneous and characterization based on long-term expansion has not been reported. To overcome the cellular heterogeneity, cell sorting approaches have been explored to isolate neural stem/precursor cells from either fresh neural tissue or established cultures. Two strategies have been developed. In one version, fluorescent proteins are used as reporters for neural precursor gene promoters. For example, Roy et al (2000) transfected human hippocampal precursor cells with DNA encoding humanized green fluorescent protein (hGFP) that was placed under the control of either the nestin enhancer or the Talpha1 tubulin promoter. When isolated and cultured *in vitro*, GFP expressing cells displayed BrdU incorporation and generated mature neurons (Roy et al., 2000). However, the BrdU incorporation was only analyzed after short-term expansion, so it is not known how long the isolated cells could be expanded. Another strategy of isolating neural precursor cells relies on the detection of specific cell surface markers. Uchida et al. reported that CD133 expressing neural precursor cells from human foetal brain tissue were able to establish neurosphere cultures and generate both neurons and glial cells *in vitro* (Uchida et al., 2000). Capela and Temple (2002) later reported the enrichment of proliferative and neurogenic precursor cells from adult mouse subventricular zone (SVZ) by isolating cells expressing LeX, a carbohydrate adhesion molecule also known as CD15 (leucocyte cluster of differentiation 15) or SSEA-1 (stage-specific embryonic antigen 1), but there has no report showing this strategy can be applied for human cells. Yet, the discovery of new cell surface markers

and the employment of cell surface makers and sorting technology hold considerable promise to isolate neural precursors from primary tissues and heterogeneous cell cultures.

2.1.2 Research aims

Our lab has reported the establishment of clonogenic mouse neural stem (NS) cell lines derived from both ES cells and foetal CNS (see Chapter 1). Here I apply this approach to human foetal CNS tissue to investigate the possibility of establishing human NS cells lines. In addition, the research aims in this part include: 1) to derive human NS cell lines from both foetal brain and spinal cord; 2) to characterize their proliferation and differentiation capacity; 3) to determine the culture requirements for both NS cell expansion and differentiation; 4) to evaluate the gene expression profile, the long-term stability, and clonogenic capacity of human NS cells; 5) to perform genetic modification to derive reporter human NS cell lines; and 6) to investigate the possibility of isolating committed neuronal progenitors by FACS.

2.2 Results

2.2.1 Derivation and expansion of adherent human NS cells

The source that I have employed to derive human NS cells is human foetal neural tissue at embryonic 50-55 days, equivalent to Carnegie stage 19-22. Human foetal cortex was carefully dissected and dissociated into single cells by incubation with Accutase. Initially, primary cells were seeded onto gelatin coated plates following protocols developed in mouse NS cell cultures (Conti et al., 2005). However, human cells proliferated very slow on gelatin substrates and displayed notable spontaneous detachment. Since I noticed that mouse NS cells show better attachment on laminin (data not shown), I plated primary human foetal cells onto laminin coated dishes in growth medium containing both EGF and

FGF2. On laminin substrate, cells readily attached and produced a morphologically heterogeneous population containing both neural precursors (Nestin+) and neurons (Tuj1+) (Fig. 2.1Aa-c). To enrich for undifferentiated neural precursor cells, on 7th day after plating, I temporarily transferred primary human cells onto gelatin coated dishes, as under these conditions, neurons and neuronal progenitors fail to survive. Seven to 10 days later, viable precursor cells were re-plated back onto laminin substrate for further expansion. The cell population was then taken over by proliferating neural precursor cells. Three weeks after (initial) plating, immunostaining indicated that the primary human culture was homogeneously Nestin positive and Tuj1 negative (Fig. 2.1Ad-f). At this stage, cultures were considered to be passage one human NS cells.

Once established, human NS cells can be expanded continuously in monolayer culture. On laminin substrate, the doubling time of human NS cells is 2-3 days, slower than mouse NS cells grown in the same condition (Conti et al., 2005; Pollard et al., 2006). I routinely split human NS cells 1:2 to 1:3 every 5-7 days. Proliferating human NS cell cultures contain both small bipolar cells and more flattened apolar cells. Time-lapse videomicroscopy reveals dynamic interconversions between these morphologies. The cells are also highly motile (Fig. 2.1Ba and Video 2.1). Some elongated cells show interkinetic nuclear migration as previously noted for mouse NS cells (Conti et al., 2005).

To investigate whether the human NS cell culture conditions can be used reproducibly, I repeated the derivation and expansion experiments using 8 independent tissue specimens, from human foetuses at embryonic day 50-55. Human NS cell lines could be generated from all specimens in which the neural tissue was well preserved. The continuous adherent procedure is more efficient than allowing primary cells to form neurospheres and subsequently isolating NS cells, as described previously (Conti et al., 2005). From one foetal brain sample, it takes, on average, one month to derive an adherent and

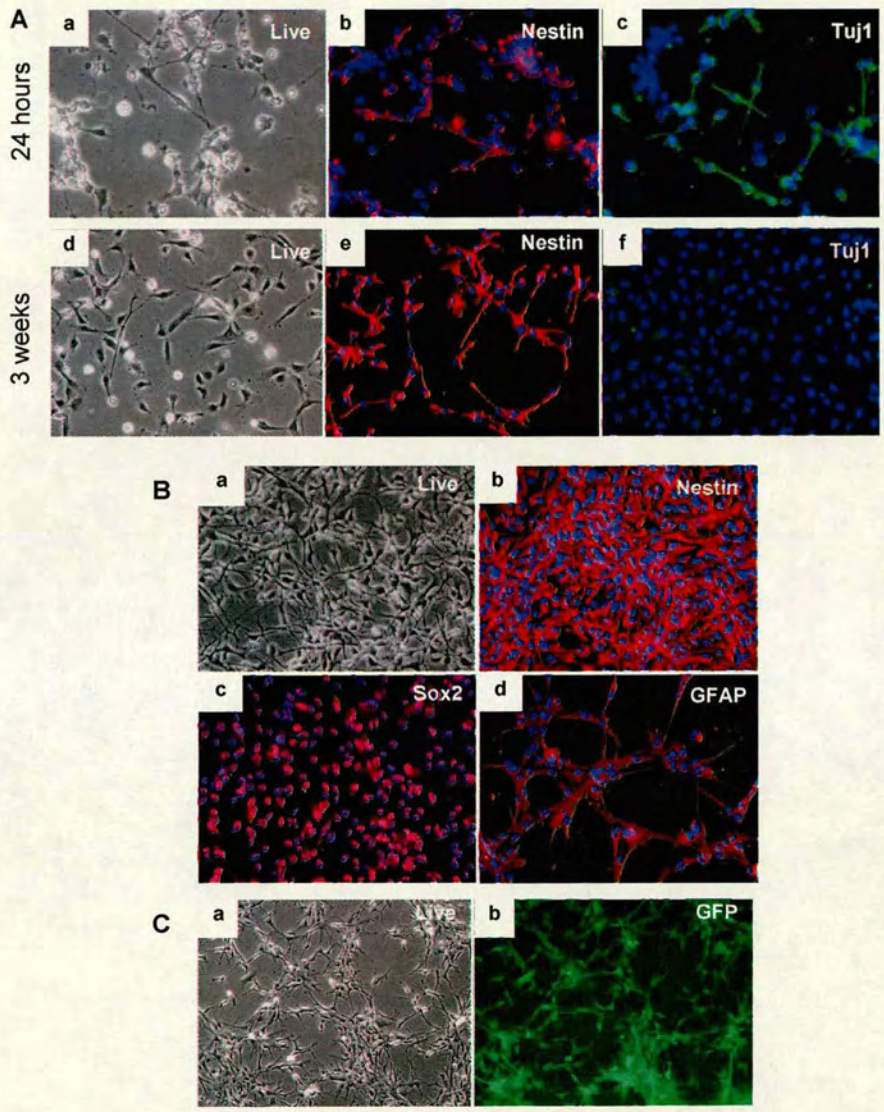

A
a
Live
b
Nestin
c
Tuj1
24 hours
d
Live
e
Nestin
f
Tuj1
3 weeks
B
a
Live
b
Nestin
c
Sox2
d
GFAP
C
a
Live
b
GFP

Figure 2.1 Derivation and expansion of human NS cells

(A) *Primary cultures.* Primary cells from human foetal forebrain were plated onto laminin coated plastic. Immunostaining performed 24 hours later indicated a heterogeneous cell population containing neural precursors (nestin+) and neurons (Tuj1+) (a-c). After 3 weeks of expansion, almost all cells were nestin positive and Tuj1 negative (d-f).

(B) *Expansion cultures.* Human NS cells can be expanded continuously in the presence of both EGF and FGF2 (a). NS cells uniformly express Nestin and Sox2 (b, c), and show weak staining with GFAP antibody (d).

(C) *Genetic modification.* CB541 human NS cell line was transfected with pCAGGFPIP plasmid DNA. GFP+ cells were purified by FACS and expanded for >3 months with retention of GFP expression (a, b).

morphologically homogeneous human NS cell population with a total number of ~2 million cells. To date, I have successfully derived 5 brain human NS cell lines, named CB192, CB516, CB525, CB541, and CB660. In addition, human NS cell lines can also be derived from foetal spinal cord using the same culture conditions. Spinal cord NS cells and brain NS cells are morphologically indistinguishable (data not shown). I have derived 4 human foetal spinal cord NS cell lines, named CB516SP, CB525SP, CB540SP, and CB660SP. To date, CB192, CB516, CB541, CB660, CB516SP, and CB660SP NS cell lines have been expanded for over one year to passage ~50. None displayed cell senescence, crisis, or spontaneous differentiation.

In expansion culture, human NS cells homogeneously express immature neural precursor markers Nestin and Sox2 (Fig. 2.1Bb, c). In contrast to mouse NS cells, human NS cells are weakly immunoreactive to anti-GFAP antibodies (Fig. 2.1Bd), consistent with the known activity of the human GFAP promoter in radial glia progenitor cells (Malatesta et al., 2000; Rakic, 2003). Real-time PCR shows that the level of GFAP mRNA expression in human NS cells is much lower than human astrocytes derived from NS cells upon serum or BMP exposure. Furthermore, human NS cells do not express the astrocyte and ependymal cell marker S100β (Fig. 2.2). Expression of neuronal markers (DCX, Tuj1, or MAP2) is not detected in expansion conditions (data not shown).

Human NS cells can be genetically modified. I transfected CB541 human NS cells with linearized pCAGGFPIP plasmid DNA by Nucleofection. ~40% of cells displayed Green Fluorescent Protein (GFP) expression 30 hours after transfection. Human NS cells with stable and readily visualised GFP expression were isolated by repeated cell sorting at 3, 6, and 9 weeks after transfection. Isolated cells were homogeneously GFP positive and could be expanded for at least 3 months (Fig. 2.1Ca, b). They retained GFP expression after differentiation into neurons or glia (data not shown).

2.2.2 Human NS cells generate neurons, astrocytes, and oligodendrocytes

Neural stem cells, as a type of tissue stem cell, are anticipated to be able to generate the three major cell types of the CNS: neurons, oligodendrocytes, and astrocytes. To assess the differentiation potential of human NS cells, I plated ~1 x 10^5 cells into poly-ornithine and laminin coated 35-mm dishes for differentiation cultures, based on protocols previously developed for mouse NS cells (Conti et al, 2005).

2.2.2.1 Neuronal differentiation

Neuronal differentiation was triggered by removing EGF from growth medium. Over 14 days, cells began to from small aggregates (still attached) and developed elongated spindle processes, accompanied by reduced proliferation and some cell death (Video 2.2). Although some cells at this stage presented neuronal morphology and Doublecortin (DCX) expression (see below), the absence of Tuj1, MAP2, or Neurofilament expression suggests these cells were immature neuronal progenitors. For further differentiation and maturation, FGF2 was withdrawn and cultures were maintained for another 2-3 weeks. By the end of 4th week of neuronal differentiation, many cells became Tuj1 positive and exhibited thin elongated processes. On the basis of 8562 cells examined in four separate experiments, 43±4.6% of cells were Tuj1 positive (Fig 2a and Video 2). The majority (76±3.7%) of Tuj1 positive neurons also express neurofilament, a marker for mature neurons (Fig 2.2b). Most non-neuronal cells (DCX/Tuj1/MAP2/Neurofilament negative) also presented extended morphology, although they retained Nestin/GFAP expression (Fig. 2.2c). On the basis of 1.2×10^6 cells scored in three separate analyses, flow cytometry indicates that 28±2.3% of intracellularly stained cells exhibited a high level of GFAP expression at the end of the differentiation culture, suggesting that a proportion of human NS cells differentiated into astrocytes (Fig. 2.2c, d). No O4 (oligodendroglial marker) expression could be detected in these conditions (data not shown). I find that all human NS cell lines

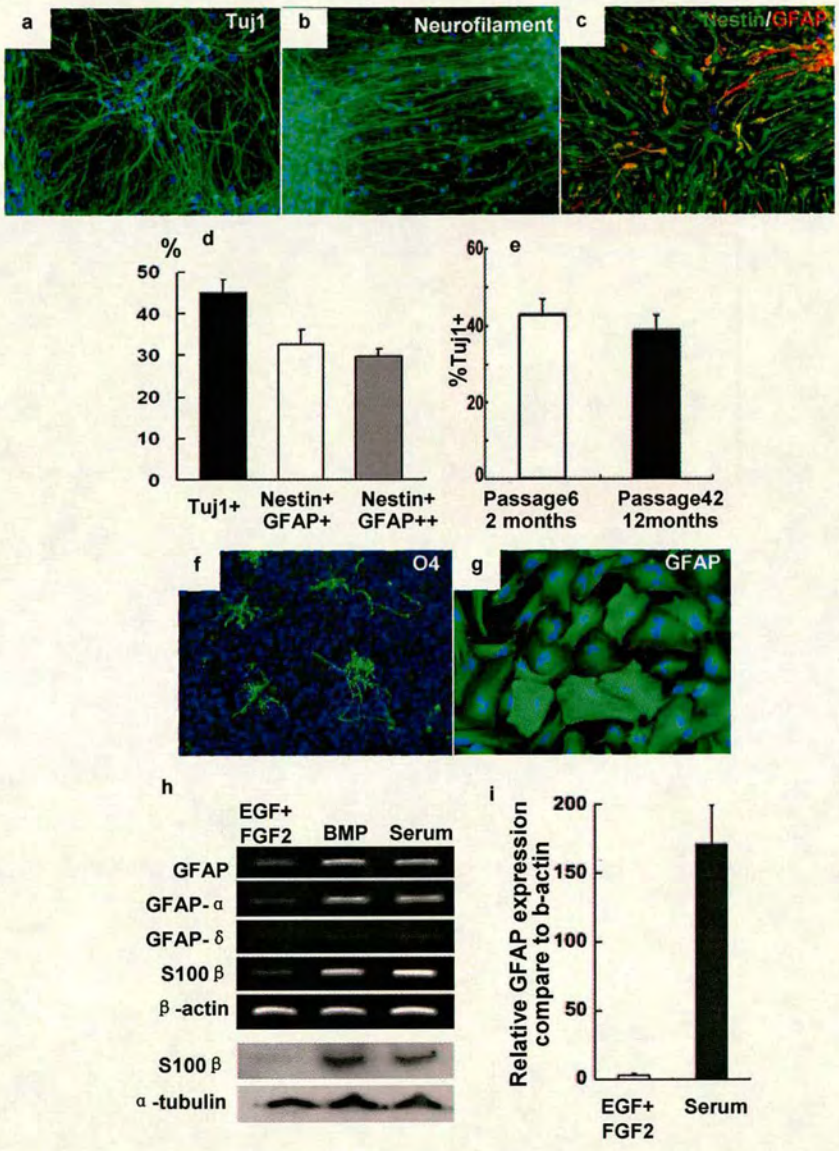

a
Tuj1
b
Neurofilament
c
Nestin/GFAP
%
d
50
40
30
20
10
0
Tuj1+
Nestin+ GFAP+
Nestin+ GFAP++
60
e
%Tuj1+
40
20
0
Passage6 2 months
Passage42 12months
f
O4
g
GFAP
h
EGF+ FGF2
BMP
Serum
GFAP
GFAP-α
GFAP-δ
S100β
β-actin
S100β
α-tubulin
i
Relative GFAP expression compare to b-actin
200
150
100
50
0
EGF+ FGF2
Serum

Figure 2.2 Human NS cells are tripotent

Over 40% of cells express neuronal marker β III-tubulin (Tuj1) and Neurofilament one month after mitogen withdrawal (a, b). Remaining cells can be stained with nestin and/or GFAP antibodies (c). Flow cytometry indicates the percentage of immunopositive cells after 30 days differentiation (d) (For flow cytometry analysis, triple staining was performed using Tuj1, Nestin, and GFAP antibodies. The percentage of immunopositive cells was analyzed by a CyAnTM ADP flow cytometer. $2x10^5$ cells were scored for each analysis, and the average percentages of each cell population were derived from three independent differentiation experiments). Human NS cells retain robust neurogenic capability after nearly one year expansion, generating ~ 40% Tuj1+ cells, similar to cells at early passages (e). When treated with PDGF, FGF2, forskolin, and T3, human NS cells are able to generate O4+ oligodendrocytes (f). Human NS cells generate a pure astrocyte population with flat sheet-like morphology upon exposure to serum or BMP4 (g). RT-PCR and real-time PCR show astrocytes express a much higher level of GFAP (GFAP α) than NS cells (h, i). RT-PCR and western blot indicate human NS cells express little or no S100 β compared with astrocytes (h).

retain stable and robust neurogenic capacity after extended expansion. For example, CB192 NS cells at passage 42 (after nearly one year expansion) were still able to generate 39±5.1% Tuj1+ cells (Fig. 2.2e).

2.2.2.2 Oligodendroglial differentiation

To generate oligodendrocytes, cells seeded on poly-ornithine/laminin coated dishes were treated with DMEM/F12 medium supplemented with N2, forskolin, FGF2, and PDGF for 2 weeks (Glaser et al., 2007). From the third week, medium was changed to DMEM/F12 medium supplemented with N2, PDGF, T3, and ascorbic acid (Glaser et al., 2007). Seven days later, PDGF was withdrawn from culture. At the end of the 5th week, 1-2% O4 positive cells with branched oligodendrocyte morphology could be detected in culture (Fig. 2.2f) along with 16±3.9% Tuj1 positive neurons (data not shown). The majority of cells remained O4 negative and Nestin positive, but up to 30±2.3% expressed NG2 (data not shown), a marker associating with oligodendroglia progenitors.

2.2.2.3 Astroglial differentiation

In the absence of EGF and FGF2, a pure astrocyte population can be derived from human NS cells after 2 weeks culture with BMP4 or serum. A time-lapse video shows that, upon exposure to serum, the cell migration and proliferation attenuate significantly (Video 2.3). Unlike NS cells, astrocytes present flat sheet-like morphology with large nuclei. They are negative for Nestin and Sox2, but they express the astroglial marker S100β and a high level of GFAP (Fig. 2.2g-i). Simply removing EGF and FGF2 from expansion medium without addition of BMP or serum also leads to NS cell differentiation into astrocytes. However, significant cell death occurs under such conditions. Recently it was reported that different types of human astrocytes may express distinct GFAP isoforms. For example, adult subventricular zone (SVZ) astroglial cells express GFAPδ, while most other astrocytes express GFAPα (Roelofs et al., 2005). In my cultures, both human foetal NS

cells and derivative astrocytes express GFAPα, but at different levels (Fig. 2.2h).

2.2.3 Culture requirements for human NS cell self-renewal

The above observations suggest that human NS cells are expandable and tripotent stem cells. I then tested a series of conditions to define the culture requirements for human NS cell self-renewal.

EGF and FGF2 are mitogens widely used in neural stem cell cultures. During derivation (the first 4 weeks after cells are plated), primary human cells attached on laminin substrate within 24 hours and started to proliferate in presence of both EGF and FGF2 (Fig. 3Aa). However, in medium containing one of the growth factors, no extended cell proliferation was observed, and NS cell lines could not be established (Fig. 2.3Ab, c). In addition to EGF and FGF2, I find laminin substrate is important for efficient human NS cell derivation. Primary cells grown on gelatin coated or uncoated dishes easily detach and tend to form neurospheres (Fig. 2.3Ad and data not shown).

During long-term expansion (>1 year), the continuous proliferation of human NS cells is also optimal on laminin substrate with both EGF and FGF2 (Fig. 2.3Ba-c). Switching laminin substrate to gelatin or uncoated plastic leads to cell detachment (Fig. 2.3Bd-f). Transfer to FGF2 only leads to neuronal differentiation accompanied by attenuated proliferation and cell death (Video 2.2). In medium containing EGF alone, neuronal and glial differentiation remain fully suppressed, but cells exhibit a slower proliferation rate with a doubling time of 3-4 days (Fig. 2.3Bg-i). When human NS cells expanded in EGF only are exposed to differentiation conditions described above, they are able to generate both neurons and glial cells. These observations mirror our previous findings with mouse NS cells, where addition of FGF is essential for initial derivation but can be dispensed with during subsequent propagation (Pollard et al., 2006).

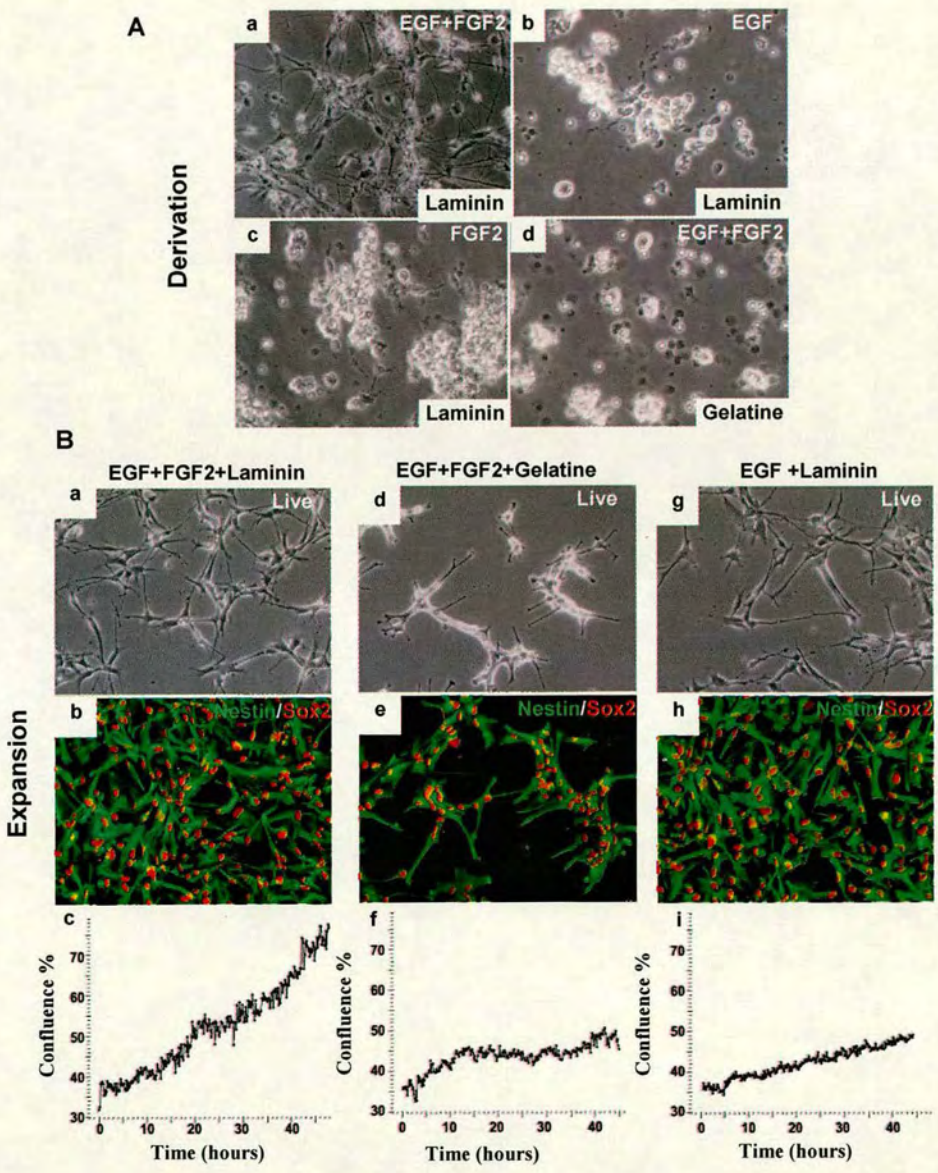

A
Derivation
a
EGF+FGF2
Laminin
b
EGF
Laminin
c
FGF2
Laminin
d
EGF+FGF2
Gelatine
B
Expansion
EGF+FGF2+Laminin
EGF+FGF2+Gelatine
EGF +Laminin
a
Live
d
Live
g
Live
b
Nestin/Sox2
e
Nestin/Sox2
h
Nestin/Sox2
c
f
i
Confluence %
Time (hours)
30
40
50
60
70
0
10
20
30
40

Figure 2.3 Characterisation of culture conditions for human NS cells

(A) *Derivation.* Efficient derivation of human NS cells requires a combination of EGF, FGF2, and laminin coating (a). No significant proliferation can be obtained in primary cultures using EGF or FGF2 alone (b, c). Primary cells tend to detach and form neurospheres on gelatine coated dishes (d).

(B) *Expansion.* In the presence of EGF and FGF2, Nestin+/Sox2+ human NS cells have a doubling time of is 2-3 days on laminin coated dishes (a-c). Replacing laminin with gelatine substrate results in significant cell detachment and reduced rate of expansion (d-f). Removing FGF2 from expansion medium leads to slower expansion, but cells retain Nestin/Sox2 expression (g-i).

Studies on human neurospheres have suggested that LIF is important for long-term stability of human progenitors (Carpenter et al., 1999; Wright et al., 2003). In order to investigate the effects of LIF on monolayer human NS cells, I performed parallel experiments throughout derivation, expansion, and differentiation procedures. At all stages, cells cultured with recombinant human LIF were not distinguishable to those without. Furthermore, all the extended passaging studies were carried out without LIF, indicating that it is not required for long-term NS cell propagation. This suggests that the reported effects of LIF on neurospheres may be indirect or only related to the neurosphere culture system. In addition to LIF, I also tested Wnt5a, Noggin, or conditioned medium in human NS cell cultures, but cultures with these supplements did not show any difference.

2.2.4 Long-term stability of clonogenic human NS cell lines

Using the culture conditions described above, human foetal NS cells can be maintained as stable cell lines. However, one may consider that progenitor cells with limited differentiation capacity could also exhibit proliferative phenotypes, and the multi-lineage derivatives in bulk culture may come from multiple types of unipotent progenitor cells. In order to determine the potency of human NS cells, I performed clonal analyses. Single human NS cells were deposited into a 96-well plate using a flow cytometry sorter. After 4 weeks expansion, 8 of 96 deposited single cells generated colonies (Fig. 2.4Aa, b). I expanded two colonies to establish clonal NS lines named CB192-1 and CB192-2. Both cell lines display extensive similarities to their parent cell line CB192. They show stable proliferation and uniform Nestin/sox2 expression in expansion medium (Fig. 2.4Ac), and both are able to generate neurons, astrocytes, and oligodendrocytes in appropriate differentiation conditions (Fig. 2.4Ad-f).

One concern in cell culture is that proliferative cell populations may undergo genetic

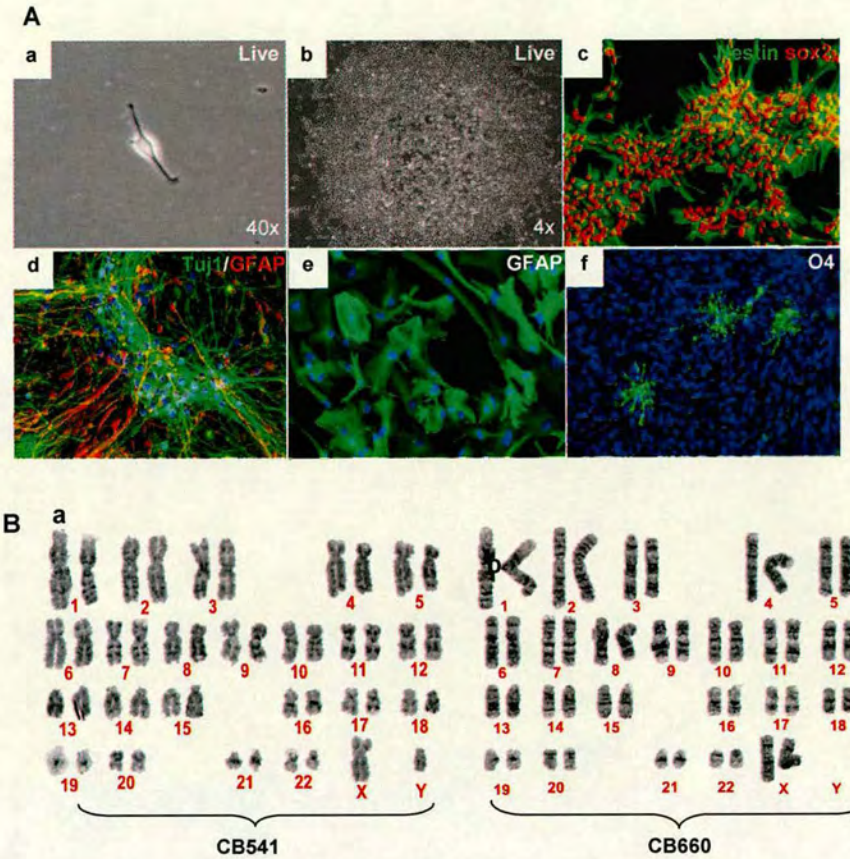

A
a
Live
40x
b
Live
4x
c
Nestin sox2
d
Tuj1/GFAP
e
GFAP
f
O4
B
a
CB541
CB660
c
TRAP products
Internal control
Glioblastoma cells
+ Δ
Passage 6 NS cells
Passage 45 NS cells
d
21.2kb
8.6kbp
6.1kbp
4.2kbp
2.7kbp
2.0kbp
+
—
Passage 6 NS cells
Passage 45 NS cells

Figure 2.4 Clonogenicity and cytogenetic stability of human NS cells

(A) *Clonal human NS cell lines.* Clonal cell lines derived from single human NS cells retain uniform nestin and sox2 expression (a-c). Clonal NS cell lines are tripotent, generating neurons, astrocytes, and oligodendrocytes in appropriate differentiation conditions (d-f).

(B) *Cytogenetic stability.* CB541 (passage 45) and CB660 (passage 42) human NS cells retain normal diploid karyotype (CB541: 46, XY; CB660: 46, XX) (a, b). A TRAP assay shows that telomerase-mediated 6-nucleotide ladder products cannot be detected in human NS cells at either early (sample 3) or late passages (sample 4), but such products are evident in human glioblastoma cells (sample 1) (c). The telomeric length of human NS cells at early (sample 3) and late passage (sample 4) is measured using a non-radioactive chemiluminescent assay. Control DNA (provided by manufacturer) and reaction buffer (no DNA) were prepared as positive (sample 1) and negative (sample 2) controls. The mean length of terminal restriction fragments (TRF) are: sample 1: 10.2kbp; sample 3: 9.8kbp; sample 4: 7.2kbp (d).

transformations, resulting in circumvention of cell cycle regulatory mechanisms and manifesting in an immortalized phenotype. Genetically immortalized cells are frequently marked by irregular/hyperplasic growth rate, abnormal chromosome numbers, and/or robust telomerase activity. In human NS cell cultures, cells display a constant doubling time of 2-3 days during long-term expansion. Their proliferation remains fully dependent on exposure to EGF and FGF2. In addition, cells retain normal diploid karyotype (46, XY for CB541 cell line and 46, XX for CB660 cell line) after over 100 generations (Fig. 2.4Ba). I find that human NS cells do not display telomerase activity (Fig. 2.4Bb), and the average length of telomeres in human NS cells shortens during expansion, from ~9.8kbp at passage 6 to ~7.2kbp at passage 45 (Fig. 2.4Bc). These findings demonstrate that human NS cells are different from tumor cells (Fig. 2.4Bb). However, it also implies that, although human NS cells are highly expandable, they may undergo replicative ageing during very extensive expansion.

2.2.5 Human NS cells exhibit similarities to radial glia and retain a subset of regional markers

An important issue to consider when characterizing stem cells *in vitro* is whether these cells have *in vivo* counterparts. Our previous study indicates mouse NS cells exhibit similarities to radial glia (Conti et al., 2005; Pollard et al., 2006), which generate both neurons and glia in the developing brain (Malatesta et al., 2000; Noctor et al., 2001; Merkle et al., 2004). Immunostaining and RT-PCR reveal that human NS cells express a set of markers including brain lipid binding protein (BLBP), 3CB2, astrocyte-specific glutamate transporter (GLAST), Vimentin, and GFAP (Fig. 2.5a-d), which are hallmarks for radial glia cells. In addition, human NS cells express neural progenitor markers including Nestin, Sox2, Pax6, Olig2, and CD133 (Prominin) (Fig. 2.5d and Fig 2.6). Sox1 is transiently expressed in mouse and human neural precursor cells derived from ES cells, but is not maintained in NS cells (Ying and Smith, 2003; Conti et al., 2005; Lowell et al.,

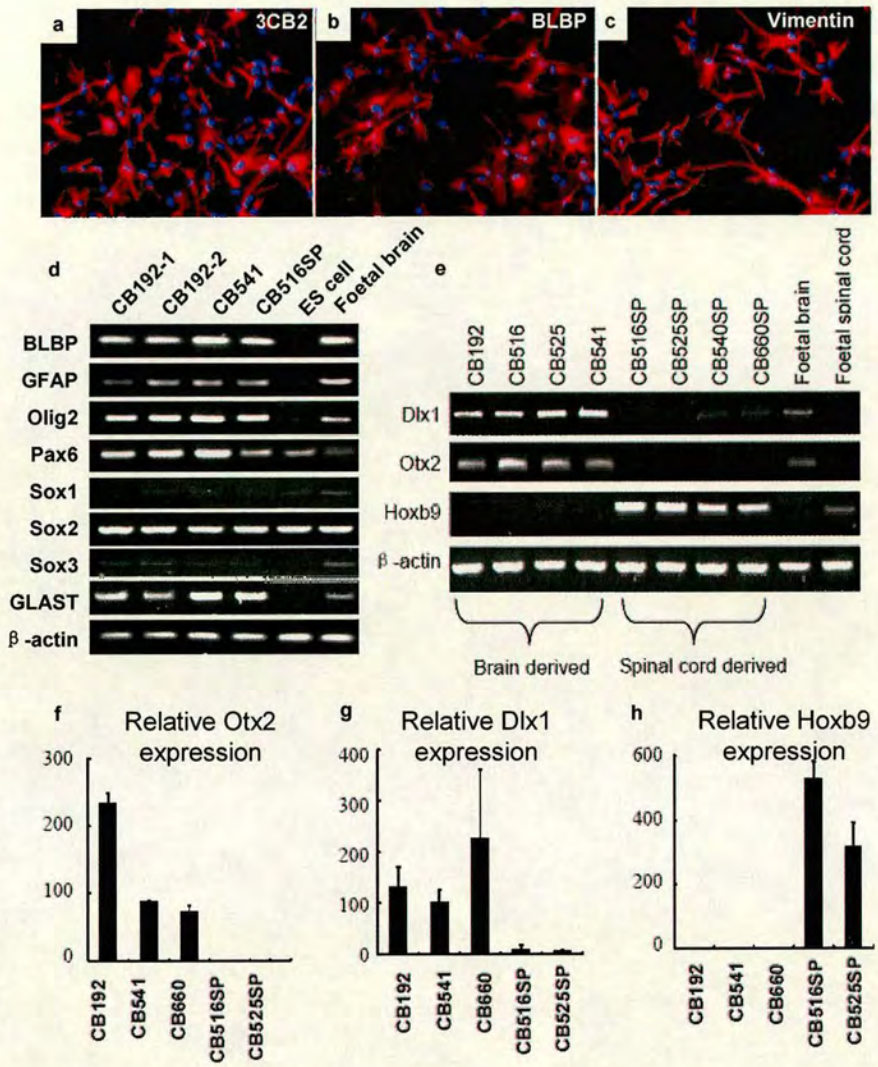


Figure 2.5 Human NS cells express radial glia markers and retain a subset of regional markers

Immunostaining shows human NS cells express 3CB2, BLBP, and Vimentin (a-c). RT-PCR indicates independent human NS cell lines express same pattern of neural precursor/radial glia markers. CB192-1 and CB192-2 are clonal lines. CB541 and CB516SP NS cells are derived from foetal brain and spinal cord respectively (d). RT-PCR and real-time PCR show human brain NS cells (CB192, CB541, CB525, CB660) express Otx2 and Dlx1 mRNA, while spinal cord cells (CB516SP, CB525SP, CB540SP, CB660SP) express Hoxb9 (e-h).

2006). Similarly, human foetal NS cells do not express Sox1.

In terms of transcription factors representing regional identities, NS cell lines derived from whole foetal forebrain express Otx2 and Dlx1 (derivations from other specific regions have not been analyzed). In contrast, cell lines derived from foetal spinal cord do not express Otx2 or Dlx1, but they express Hoxb9 (Fig. 2.5e-h), suggesting some level of regional identity may be retained in NS cells from different sources. Expression of other regional markers Emx1/2, Gsh1/2, and Dlx2 is not detected in either brain or spinal cord human NS cells.

2.2.6 Cell population transitions during neuronal differentiation

Early stages of neuronal differentiation from human neurospheres are difficult to visualise and study due to multicellularity and heterogeneity. Adherent human NS cells allow direct inspection of both stem cells and their differentiating progeny. I employed videomicroscopy, time-course immunostaining, and RT-PCR to track the cell population transitions during neuronal differentiation and begin to define the properties of intermediate neuronal progenitor cells.

As described above, the protocol for *in vitro* neuronal differentiation of human NS cells consists of 2 weeks of FGF2 induction followed by 2-3 weeks of maturation in basal medium without growth factor (Fig. 2.6a-c and Video 2.2). During weeks 1-2, immunostaining shows expression of neural stem cell surface marker CD133 decreases sharply, while the expression of polysialylated neural adhesion cell molecule (PSA-NCAM) and DCX emerge (Fig. 2.6d, e, g, h). During weeks 3-4, Tuj1+ cells appear soon after FGF2 withdrawal, and Tuj1 staining overlaps with PSA-NCAM or DCX staining (Fig. 2.6f, i). Nestin+ cells persist throughout the differentiation procedure, and they constitute the majority of non-neuronal cells (Tuj/DCX/PSA-NCAM negative) at

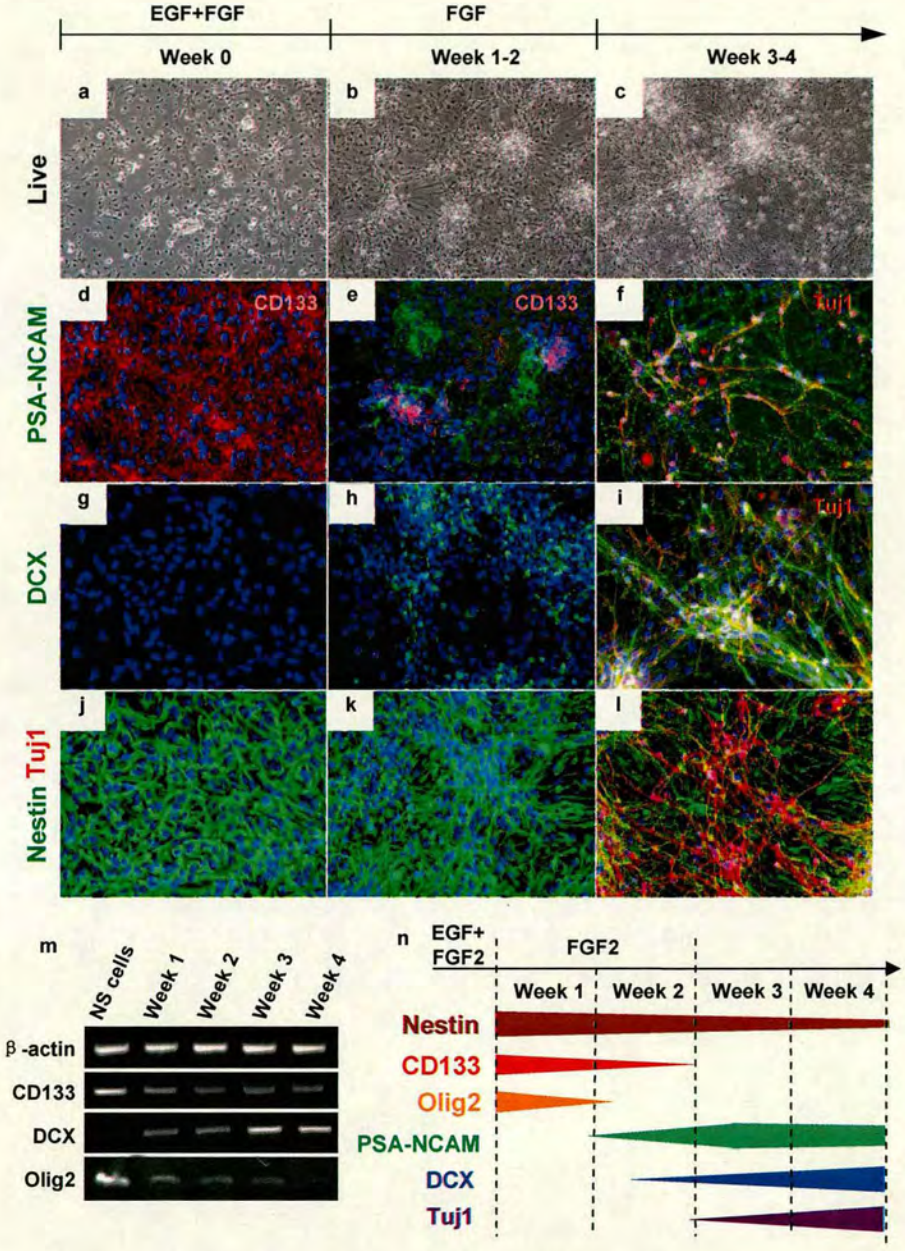


Figure 2.6 Time-course studies of neuronal differentiation

Human NS cells undergo neuronal differentiation upon sequential withdrawal, first of EGF and then of FGF (a-m). Figure 6n summarizes the qualitative marker changes over 4-week differentiation.

week 4 (Fig. 2.6j-l). RT-PCR confirms the decrease of CD133 and increase of DCX during neuronal differentiation (Fig. 2.6m). In addition, Olig2 expression also decreases soon after the onset of differentiation (Fig. 2.6m). Fig. 2.6n summarizes the marker expression changes during the 4-week neuronal differentiation.

2.2.7 Isolation of PSA-NCAM positive neuronal progenitors from differentiating NS cultures

Although human NS cells retain neurogenic capacity over long-term *in vitro* expansion, only ~40% of cells differentiate into neurons using the current protocol. This heterogeneity complicates global characterization of the neurons. PSA-NCAM is a cell surface marker that appears to be expressed only by cells undergoing neuronal differentiation (see above). This suggests PSA-NCAM could be a candidate marker for isolating neuronal progenitors from neuronal differentiation cultures. I therefore assessed whether neuronal progenitors or neurons may be purified from differentiation cultures using PSA-NCAM antibody staining and subsequent Fluorescence Activated Cell Sorting (FACS)

Although neuronal differentiation cultures at later time point (week 3-4) contain higher percentage of PSA-NCAM positive cells, the viability of cells after FACS is low. I therefore performed flow cytometric sorting with CB660 and CB660SP NS cells on day 14 of FGF2 only cultures (Fig. 2.7a). Flow cytometry indicated 6.8±1.1% of cells were PSA-NCAM+ at this time point, and most of them (82.4±5.2% of the PSA-NCAM positive cells) retained CD133 expression. 40.9±3.3% of cells remained CD133 positive and PSA-NCAM negative. 51.8±4.2% of cells could not be stained with either CD133 or PSA-NCAM antibodies. I sorted these four cell populations and re-plated them on laminin coated dishes for further differentiation (cultures without mitogen) (Fig. 2.7a). Two weeks later, only the PSA-NCAM+ cell population generated Tuj1+ neurons. Although the

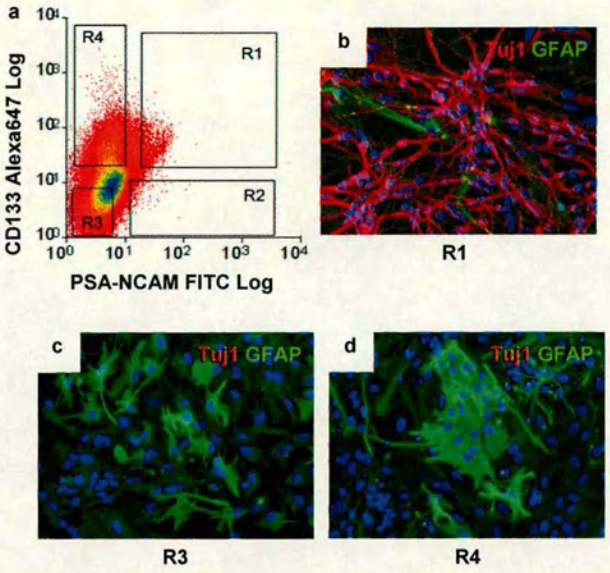


Figure 2.7 Isolating neuronal progenitors in differentiation culture

On day 14 of neuronal differentiation (NS cells treated with FGF2 only), live cells were stained with anti-PSA-NCAM and CD133 antibodies and sorted using gates illustrated in 7a (R1-R4). Sorted cells were re-plated onto laminin coated dishes for further differentiation (without mitogen). Two weeks later, only PSA-NCAM+ cells (R1) generated Tuj1+ neurons, although the culture still contained GFAP positive cells (b) (Cells from R2 did not survive well after re-plating, probably due to limited number of cells). Most PSA-NCAM negative cells (R3 and R4) differentiated into astrocytes with high level of GFAP expression (c, d).

cultures still contained GFAP and Nestin expressing cells at the end of differentiation, the percentage of Tuj1+ neurons generated from PSA-NCAM+ cells could reach 60-70% (Fig. 2.7b). In contrast, most PSA-NCAM negative cells differentiated into astrocytes with strong GFAP staining (Fig. 2.7c, d). This indicates that PSA-NCAM can be used as a maker to enrich viable neuronal progenitors.

2.2.8 Human NS generate Calretinin positive neurons

To clarify which type(s) of neurons may be generated from human NS cells in culture, I stained derived neurons with different neuronal subtype markers. In both brain and spinal cord cell cultures, ~5% of Tuj1+ neurons displayed Calretinin expression (Fig. 2.8a, b). Although I managed to derive Tuj1+ neurons with modified protocols by adding Shh, FGF8, and/or B27 (either with or without retinol acetate) into differentiation medium, I did not detect specific neuronal markers other than Calretinin. The neuronal markers we have tested so far by antibody staining and RT-PCR include: Darpp32 Somatostatin, Parvalbumin, Calbindin, Neuropeptide Y, GAD67, GABA, Islet-1, TH, and ChAT.

In order to examine whether human NS cells generate functional neurons, I sent CB541 and CB660 human NS cells to the University of Pavia for electrophysiological analysis. Dr. Mauro Toselli and Dr. Gerardo Biella performed patch-clamp recordings after neuronal differentiation following my protocol. Their results indicate that neurons derived from human NS cells are electrophysiologicaly active and display typical properties for mature neurons (see detailed results and methods in Appendix I).

2.3 Discussion

Cultured neural stem cells hold considerable promise in both biological research and

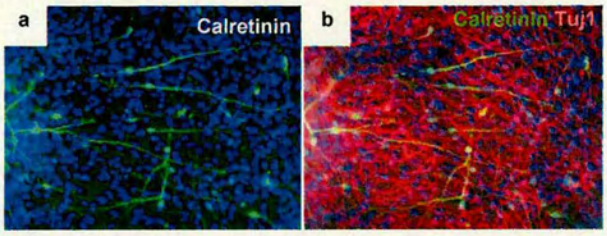


Figure 2.8 Human NS cells generate Calretinin positive neurons

After 4 weeks neuronal differentiation, immunostaining show ~5% of Tuj1+ neurons express interneuron marker Calretinin (a, b)

potential cell-replacement therapies. Current studies of human neural cells largely rely on primary neurosphere cultures or genetic immortalization. However, as mentioned in Chapter 1, the nature of cellular heterogeneity in neurospheres prevents direct interrogation of sphere-forming cell populations (Suslov et al., 2002; Reynolds and Rietze, 2005), and one cannot infer the existence of neural stem cells in individual neurospheres, even if they are derived in clonal conditions (Reynolds and Reitze, 2005; Singec et al., 2006). Genetic immortalization allows extensive expansion and clonal analysis of cultured human neural progenitors (Flax et al., 1998; Villa et al., 2000). However, a major concern of perpetuated cells is that oncogenes may subvert normal regulation of cell cycle, apoptosis, and differentiation, so that perpetuated cells may not reflect fully the properties of original cell populations. In addition, continuous oncogene expression in immortalized cells prohibits use of these cells for cell therapy.

In this chapter, I described adherent human foetal NS cells derived from both brain and spinal cord that 1) express appropriate markers of neural stem cells; 2) show clonogenicity and long-term stability without genetic immortalization; 3) exhibit tripotent differentiation potential, generating oligodendrocyte, astrocytes, and mature neurons even after prolonged expansion; and 4) are readily derived and expanded in fully defined culture conditions. These findings establish that human NS cell lines are self-renewing and tri-potent, human neural stem cell population *in vitro*.

Human NS cells display similar culture requirements as mouse NS cells (Conti et al., 2005; Pollard et al., 2006). They both propagate in the presence of EGF and FGF2, under which conditions differentiation into neurons and glia is fully suppressed. Both human and mouse NS cells can be expanded with addition of EGF only (Pollard et al., 2006), suggesting EGF is the major mitogen for NS cell self-renewal, although a contribution of autocrine FGF is not excluded. However, neither human nor mouse primary cells produce

stable cell lines unless they are exposed to FGF2 during the first 2-4 weeks after plating. A possible contributing factor is that FGF2 may induce EGF responsiveness in NS cells in the first instance, which is consistent with previous observations on other rodent neural progenitors (Ciccolini and Svendsen, 1998; Santa-Olalla and Covarrubias, 1999).

My observations indicate that LIF is not required for the long-term stability of human NS cells. Human NS cells derived and expanded without LIF did not show cell crisis or senescence. This suggests the reported species specific effects of LIF in human neurosphere cultures could be indirect. It is possible that LIF may induce glial differentiation of progenitors (Bonni et al., 1997; Nakashima et al., 1999) that subsequently produce factor(s) that feedback on the progenitors to promote cell proliferation.

I find that laminin substrate is crucial for mouse/human NS cell neuronal differentiation (Conti et al., 2005) and optimal for human NS cell propagation. This indicates laminin may play important roles in regulating neural cell behaviour. Indeed, mammalian laminin has been shown to present in neural stem cell niches including embryonic periventricular regions (Liesi, 1985) and the adult SVZ (Mercier et al., 2002). Rodent models with laminin receptor mutations display cortical abnormalities in association with pathological features of CNS diseases (Colognato et al., 2005). Interestingly, while mouse NS cells also require laminin to under neuronal differentiation, they can be expanded well in vitro on gelatin (Conti et al., 2005). Gelatin is a heterogeneous mixture of water-soluble proteins of high average molecular weights present in collagen. The proteins are extracted by boiling skin, tendons, ligaments, bones, etc. in water. Type A gelatin is derived from acid-cured tissue and Type B gelatin is derived from lime-cured tissue. Applications using gelatin include coating cell culture plates to improve cell attachment for a variety of cell types. Moreover, as a biocompatible polymer, gelatin has been used as a delivery vehicle

for the release of bioactive molecules and in the generation of scaffolds for tissue engineering applications (Huang et al., 2005; Young et al., 2005) . It is not clear whether gelatin have biological effects on cultured neural stem cells other than a coating substrate or scaffold. Yet, one may speculate that cultured neural stem cells (either with or without coating substrates) may not behave in the same way as stem cell population *in vivo,* which are exposed to a complex microenvironment. In other words, although neural stem cells can proliferate and differentiate *in vitro*, they may not retain normal functions.

Human and mouse NS cells exhibit similar expression of transcription factors. They express neural precursor markers including Nestin, Vimentin, Sox2, Pax6, and Olig2; radial glia markers 3CB2, BLBP, and Glast; and regional markers Otx2 and Hoxb9 (Conti, et al., 2005; Pollard, et al., 2006; and data not shown). I speculate that NS cells may represent a generic mammalian neural stem cell population *in vitro*. In this context, it should be considered that the artificial nature of culture environments may result in unique cell populations *in vitro*. Therefore, NS cells do not necessarily have direct *in vivo* counterparts. In fact, the combination of transcription factor expression in mouse NS cells is not routinely observed during normal development (Pollard et al., 2006).

Although human NS cells are highly expandable, their telomeric length shortens during expansion due to the absence of telomerase, which is consistent with earlier studies on human neurospheres (Ostenfeld et al., 2000). This implies human NS cells may undergo replicative ageing like most other somatic cells (Allsopp et al., 1992). However, recent studies have noted that progressive telomeric shortening is not necessarily a constant function of cell division. For example, several growth factors, including FGF2, may maintain telomeres of human stem cells without upregulation of telomerase activity (Yanada et al., 2006). In addition, it was reported that telomere shortening may not correlate with a cell's replicative history in a straightforward or quantitative manner

(Hodes, 1999; Villa et al., 2004). Further studies are required to determine whether telomere length stabilises or shortens upon more extended human NS cell cultures.

I demonstrate that human NS cells are amenable to genetic modification and their response to pharmacological agents or recombinant proteins can be directly inspected by videomicroscopy and immunostaining. These characteristics enable NS cells to be applied as a model system for both developmental and pharmaceutical research. In fact, mouse NS cells have been utilized in studies on cell reprogramming (Blelloch et al., 2006; Silva et al., 2006) to investigate epigenetic restrictions and commitment. My data also show that human NS cells generate mature and functional neurons *in vitro*, defined by both marker expression and electrophysiological attributes (see Appendix I). Although it seems that human NS cells have limited capacity to produce multiple neuronal subtypes *in vitro*, it is possible that particular culture conditions or glial support are required for generation of specific neuronal types. I find PSA-NCAM is a promising cell surface marker that can be used to isolate neuronal progenitors. Enriched neuronal progenitors may enable more detailed characterization of neuronal differentiation *in vitro.* They may also constitute a promising cell source for potential neuroregenerative medicine.

In conclusion, human foetal NS cell lines represent a genetically normal self-renewing and tripotent human neural stem cell population *in vitro*. They can be applied to investigate fundamental questions in stem cell and developmental neurobiology. They may also serve as a scaleable source of human neurons and glia cells for genetic and pharmaceutical screening and possibly for cell-replacement therapies. It will be important to determine NS cell differentiation capacity to generate different neuronal subtypes *in vitro* and *in vivo*, and whether or how they may contribute to functional reconstruction in disease models. It should also be informative to investigate the relatedness of human NS cells to brain tumour initiating cells (Singh et al., 2004).

2.4 Materials and method

2.4.1 Tissue culture materials and reagents

2.4.1.1 Plastic and centrifuge

All tissue culture flasks, plates, and dishes were from IWAKI. 50ml and 15ml centrifuge tubes were from Corning Incorporated. 20ml universal tubes were supplied by Nunc. All centrifugations were carried out at 1400rpm in an Eppendorf 5702 Centrifuge unless stated otherwise.

2.4.1.2 NS basal medium

Two forms of NS basal medium were used in this study: Euromed-N (formerly NS-A) medium from Euroclone and RHB-basal medium from Stem Cell Sciences Ltd. Both are basal media with a formulation similar to DMEM. I supplemented basal medium with N-2 supplement (below) and 2mM L-glutamine (Invitrogen) (RHB-basal already contains L-glutamine). Growth factors were directly added to flasks/plates immediately prior to use to achieve the final NS cell expansion media.

2.4.1.3 EGF and FGF2

Recombinant mouse EGF (Peprotech) and human FGF-2 (Peprotech) were re-suspended in PBS and stored at -20°C as 20µl aliquots. EGF and FGF-2 were added directly to flasks and plates when required. Once thawed, each aliquot was used within 1 week stored at 4°C.

2.4.1.4 N2

The N2 supplement in this study has a modified formulation (Ying and Smith, 2003). It contains bovine serum albumin and higher dose of insulin. This formulation increases attachment and survival of neural cells. Batches of N2 can be stored in aliquots at -20oC for no longer than three weeks.

Stock solutions to prepare N2 aliquots are:

a) Insulin 25mg/ml (Sigma), dissolve 100mg/4ml 0.01M sterile filtered HCl. Insulin should be re-suspended overnight at 4°C.

b) Apo-transferrin 100mg/ml (Sigma), dissolve 500mg/5ml sterile filtered H_20.

c) BSA 75mg/ml, dissolve in sterile PBS.

d) Progesterone 0.6mg/ml (Sigma), dissolve 6mg/10ml Ethanol, then filter sterilize.

e) Putrescine 160mg/ml (Sigma), dissolve 1.6g/10ml H_2O, then filter sterilize.

f) Na Selenite 3mM (Sigma), dissolve 2.59mg/5ml H_20, then filter sterilise.

g) DMEM:F12 (- Glutamine) (Gibco).

These stocks were stored at -20oC. I routinely prepared 40ml of N-2 supplement as follows:

h) DMEM:F12 medium: 27.5ml

i) BSA stock: 4ml

j) Insulin stock 4ml (add 200µl at a time to prevent precipitation)

k) Apo-transferrin: 4ml

l) Sodium Selenite: 40µl

m) Putrescine: 400µl

n) Progesterone: 132µl

2.4.1.5 B27 supplement

B27 supplement is obtained from Invitrogen. 1ml B27 aliquots were stored at -20 °C, and use 1:50 in culture medium. Unless specified, B27 containing retinyl acetate was present in all experiments.

The constituents of B27 supplement are listed below (Brewer et al., 1993; Brewer, 1995) :

Table 2.1 Ingredients of B27 supplement

Biotin	Retinyl acetate
L-carnitine	Selenium
Corticosterone	T3 (triodo-1 -thyronine)
Ethanolamine	DL-a-tocopherol (vitamin E)
D(+)-galactose	DL-a-tocopherol acetate
Glutathione (reduced)	Albumin, bovine
Linoleic acid	Catalase
Linolcnic acid	Insulin
Progesterone	Superoxide dismutasc
Putrescine	Transferrin

2.4.1.6 Laminin

Laminin (Sigma) were stored at -20°C as 50μl aliquots.

2.4.1.7 Poly-ornithine/laminin coated dishes

To prepare Laminin coated dished, laminin was diluted at 1:100 in PBS (10μl/ml final). Appropriate volume of laminin solution was added into flasks or dishes, followed by incubation at 37°C for at least 24 hours (less than 24 hours).

The volume of laminin solution for different size of dishes and flasks are listed below:

Table 2.2 Volume of coating solution for culture plates and flasks

Plate/flask	Volume of laminin solution
1 well of 96-well plate	50ul
1 well of 24-well plate	150μl
1 well of 12-well plate	250ul
1 well of 6-well plate	1ml
T-25 flask	2ml
T-75 flask	5ml

To prepare poly-ornithine/laminin double coated dishes, I first coated culture plastic with 0.01% poly-L-ornithine solution (Sigma) for at least 20mins at 37°C. Afterwards, I washed plastic three times with 1x PBS, and performed laminin coating as above.

2.4.1.8 Gelatine stock and gelatine coating

I prepared 1 % gelatine (Sigma) solutions in UHP water. Autoclaved aliquots were stored at 4°C. To prepare the 0.1% working solution, 1% gelatine was warmed to 37°C and diluted 1:10 in sterile PBS. 0.1% gelatine can be stored for up to two weeks at 4°C. For culture and differentiation, plates and flasks were coated with a 0.1% gelatine solution for at least 30mins at 37°C. Gelatine was aspirated prior to use.

2.4.1.9 LIF

LIF was prepared by transient expression of human LIF expression plasmids in COS-7 cells using the method described in (Smith et al. 1990). Serial dilutions of supernatant were tested on mouse ES cells plated in 24-well plates. Based on the minimal requirement to suppress mouse ES cell differentiation, a 100-fold or higher does was used in human

NS cultures.

2.4.1.10 Accutase

Accutase solution (Sigma) was stored at -20 °C up to 6 months, or 1 month at 4°C. I routinely used Accutase to harvest adherent NS cells. 1-2 minutes incubation with Accutase at 37°C could easily detach all cells. When cells were detached, the cell suspension was diluted with 4 times volume of serum-free medium followed by centrifugations.

2.4.2 Human foetal tissue.

All studies with human tissue were performed under Ethical Approval from the Lothian Healthcare Trust using tissue donated with informed consent after elective termination of pregnancy. Human foetal brain (mainly cortex) and spinal cord tissue were collected from foetal tissue at embryonic day 50-55, equivalent to Carnegie stage 19-22. Foetal tissue was kept on ice before dissection in either Neurobasal medium (Invitrogen) or 10% sucrose in sterilized PBS. Tissue can be kept in such condition for a couple of hours, but it is important to commence derivation as soon as possible.

2.4.3 Dissection and dissociation

To dissect brain and spinal cord, foetal tissue was moved into 100mm tissue culture dish in ~20ml Neurobasal medium. It is better to use phenol red free media for a better vision under microscope. Foetal brain and spinal cord were carefully dissected and without outer membranes. Dissected tissue samples were transferred to a clean dish and cut into pieces as small as possible. Tissue samples were then incubated with a mix of PBS and Accutase (1:1) at 37°C for 5 minutes and mechanically dissociated into single cells. Cells were washed and re-suspended in expansion medium before plating.

2.4.4 Primary culture

In primary cultures, single cell suspensions were plated onto laminin coated T25 flask in expansion medium. The expansion medium comprised Euromed-N or RHB basal medium, L-glutamine (2mM final, Gibco), modified N2 supplement, B27 (20ml/L final), Penicillin-Streptomycin (10ml/L final, Sigma), and 10ng/ml of both mouse EGF (Peprotech) and human FGF-2 (Peprotech). Medium was changed every two days. After one week, cells were transferred onto 0.1% gelatine coated flask in expansion medium for purification. 7-10 days later, when neurons were eliminated, cells were plated back onto laminin substrate for further expansion. From one foetal brain or spinal cord, it took 3-4 weeks to derive a morphological homogeneous human NS cell population, with a total number of ~2 million cells.

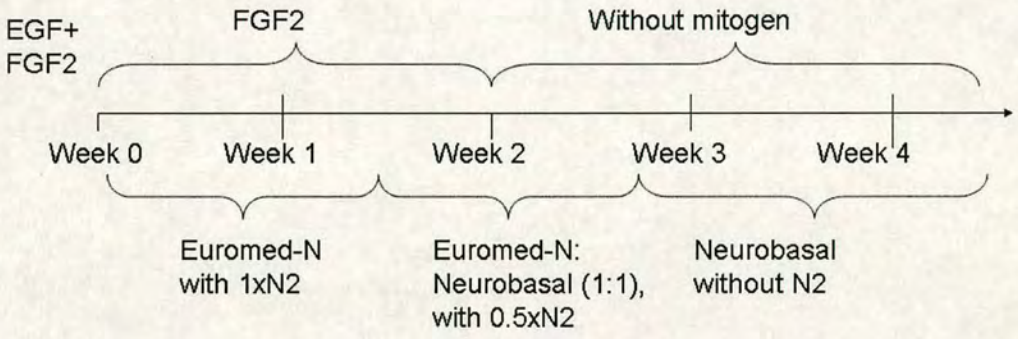
2.4.5 Expansion culture

Human NS cells could be continuously grown on laminin coated flasks/plates in expansion media. Once cells became confluent, they were harvested using Accutase and split at the ratio of 1:2. Human NS cells could easily be detached after 1 minute incubation with Accutase followed by gently tapping, and a short time exposure to Accutase helped human NS cells recover better after re-plating. A relatively high density was also beneficial for the survival and proliferation of human NS cells. Immunostaining with Nestin and sox2 antibodies was performed to confirm a homogeneous NS cell population.

2.4.6 Neuronal differentiation

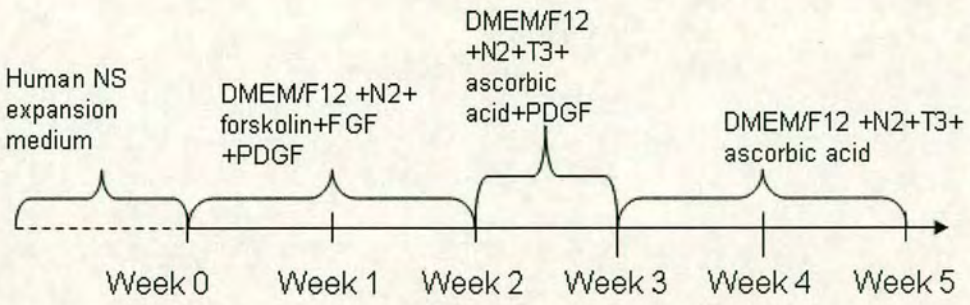
For neuronal differentiation, $1\text{-}2\text{x}10^5$ human NS cells were plated into each well of poly-ornithine and laminin coated 12- well plate in expansion medium for 24 hours. Neuronal differentiation was triggered by removing of EGF from expansion media. After 10 days, culture medium was changed to Euromed-N mixed with Neurobasal medium in a

ratio of 1:1 supplemented with N2 (0.5X), B27 (1X), and FGF2 (10ng/ml). 4 days later, FGF2 was withdrawn from medium, and after another 4 days, medium was switched to Neurobasal media supplemented with B27 (1X) and BDNF (10ng/ml, R&D systems) without N2 or FGF-2 for 10-20 days. During the whole progress of neuronal differentiation, 1/2 volumes of media were replaced every 3 days. The diagram below summarizes the differentiation protocol.



2.4.7 Oligodendroglial differentiation

To derive oligodendrocytes, 1-2x10^5 human NS cells were plated onto poly-ornithine and laminin coated plastic in expansion medium for 24-48 hours. Medium was then changed to DMEM/F12 medium supplemented with N2 (1x), forskolin (10nM), FGF2 (10ng/ml) and PDGF (10ng/ml, R&D Systems) for 14 days. From day 15, medium was switched to DMEM/F12 supplemented with N2 (1x), T3 (30ng/ml), ascorbic acid (200μM), and PDGF (10ng/ml). PDGF was withdrawn from culture on day 22 to allow maturation. O4 positive cells could be detected after a total 5-week differentiation culture. The diagram below summarizes the differentiation protocol.



2.4.8 Astroglial differentiation

In order to derive astrocytes, human NS cells were treated with 3% serum in Euromed-N or RHB-basal medium without EGF or FGF2. A pure astrocyte population could be derived after 2-3 weeks in this condition.

2.4.9 Clonal NS cell lines

To generate clonal cell lines, human NS cells were detached and suspended in expansion medium. Single cells were plated onto laminin coated 96 microwell plates by FACS deposition. It took ~4 weeks for single cells to form small colonies, which could be further expanded as clonal cell lines. In clonal assay, expansion medium was renewed by 50% change every 3 days, and cells were passaged once a month.

2.4.10 Freezing and thawing cells

To freeze cells, cultures were detached, centrifuged, and re-suspended in serum-free medium with 10% DMSO. A 60-90% confluent T25 flask was usually frozen down into 1 vial, and one T75 flask into 3 vials. Cells were frozen at 0.5ml per cryotube (Nunc) at -80°C overnight, then transferred to liquid nitrogen for long term storage.

Cells were thawed by rapidly bringing the vial to 37°C and then transferring the cells to

10ml of pre-warmed growth medium. Cells were centrifuged and plated in appropriate culture conditions.

2.4.11 Immunostaining

For immunostaining, cells were fixed with 4% PFA for 15 minutes, followed by 30 minutes incubation with BLOCK solution at room temperature. 100ml BLOCK solution contained 97ml PBS, 3ml goat or donkey serum, and 0.1% Triton-X100. After incubation with primary antibodies (2 hours at room temperature or overnight at 4°C, I used Alexa-Fluor secondary conjugates (Invitrogen) and DAPI (Sigma) to visualize the staining. Primary antibodies were used as following dilutions: Nestin (1:500, R&D Systems), Sox2 (1:400, Chemicon), GFAP (1:300, Millipore), Tuj1 (1:200, Covance), O4 (1:100, R&D Systems), 3CB2 (1:20, DSHB), BLBP (1:500, Abcam), Vimentin (1:20, DSHB), CD133 (1:10, Miltenyi Biotec), CD15 (1:10, Miltenyi Biotec); PSA-NCAM (1:200, Millipore), DCX (1:300, Cell Signaling), Neurofilament (1:300, Millipore), and Calretinin (1:200, Santa Cruz biotech.).

CD133, CD15, CD44, PSA-NCAM, and O4 staining were performed on live cells. For live staining, cells were incubated with primary antibody in serum-free Euromed-N or RHB-basal medium for 15 minutes at room temperatures. The staining was visualized by Alexa-Fluor secondary conjugates after 10 minutes incubation at room temperature. Cells could be fixed and then stained with other antibodies.

2.4.12 RT-PCR

I used RNeasy kit (Qiagen) to extract total RNA and Superscript III (Invitrogen) to prepare cDNA. The procedures followed the instructions provided by manufacturers. An additional DNase step was performed on the total RNA to remove any traces of genomic DNA.

Each PCR reaction contained a final concentration of the following: 200μM dNTP mix, 1μM of each primer, 100ng template DNA, appropriate dilution of PCR reaction buffer and 1 unit of Taq DNA polymerase. The reaction condition was set to 30 cycles for all markers except β-actin (25 cycles). Primer sequences and product sizes are listed below.

Table 2.3 Primers used for RT-PCR (human)

Gene	Forward primer	Reverse primer	Size (bp)
β-actin	GTC TTC CCC TCC ATC GTG	AGG TGT GGT GCC AGA TTT TC	181
BLBP	CCA GCT GGG AGA AGA GTT TG	CTC ATA GTG GCG AAC AGC AA	196
cd133	CAG AGT ACA ACG CCA AAC CA	AAA TCA CGA TGA GGG TCA GC	245
DCX	GAC AGC CCA CTC TTT TGA GC	TGG GTT TCC CTT CAT GAC TC	229
cd44	GGC TTT CAA TAG CAC CTT GC	ACA CCC CTG TGT TGT TTG CT-3	152
dlx1	TAC AGC TCA GCC TCG TCC TT	ACT TGG ATC GCT TGT TTT GG	179
gfap	GAA GCT CCA GGA TGA AAC CA	ACC TCC TCC TCG TGG ATC TT	165
glast	CTC ACA GTC ACC GCT GTC AT	CCA TCT TCC CTG ATG CCT TA	202
hoxb9	TAA TCA AAG ACC CGG CTA CG	CTA CGG TCC CTG GTG AGG TA	198
olig2	CAG AAG CGC TGA TGG TCA TA	TCG GCA GTT TTG GGT TAT TC	208
otx2	AGA GGA GGT GGC ACT GAA AA	ATT GGC CAC TTG TTC CAC TC	188
pax6	GGG CAA TCG GTG GTA GTA AA	CTA GCC AGG TTG CGA AGA AC	190
s100β	AGGGAGACAAGCACAAGCTG	AGGAAAGGTTTGGCTGCTTT	241
sox1	AAT CCC CTC TCA GAC GGT G	TTG ATG CAT TTT GGG GGT AT	224
sox2	GCC GAG TGG AAA CTT TTG TCG	GCA GCG TGT ACT TAT CCT TCT T	154
sox3	CCA AGG AGT GAA TGG GAG AA	AGA TCA CGG CAG AAA TCA CC	248

2.4.13 Telomerase activity, telomeric length, and karyotype analyses

Non-radioactive analyses for telomerase activity and telomeric length were performed

using appropriate kits with supplied protocols (Roche). For karyotype assay, NS cells were treated with 5ml 0.56% KCl for 15 minutes, fixed in acetic acid:methanol (1:3), spread onto glass slides, and stained with Giemsa solution.

The metaphase spreads were analyzed by Dr. Georgina Parkin and Dr. Lionel Willatt in Cytogenetics Laboratories, Regional Genetics Laboratories, Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK

2.4.14 Western Blots

Cell extracts (50 ul) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% NuPage gel, Invitrogen) and electrophoretically transferred to a polyvinylidene difluoride membrane (Amersham). The membrane was incubated with 5% nonfat dry milk at 4 oC overnight and then probed with the respective antibody (anti-αtubulin, Abcam, 1:2000; anti-S100β, Santa Cruz Biotech, 1:1000). After three washes, the membrane was incubated with 1:5000 dilution of the appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature. Washed blots were subjected to enhanced chemiluminescence.

2.4.15 Derivation of reporter NS cell line

I transfected passage6 CB541 human NS cells with ScaI linearized pCAGGFPIP plasmid DNA (Clontech) using Nucleofector (Amaxa Biosystems, program A-033). GFP expressing cells were sorted by FACS 3, 6, and 9 weeks after transfection. Isolated GFP positive cells were expanded to establish a reporter NS cell line.

2.4.16 Growth curves and time-lapse videomicroscopy

I prepared growth curves and time-lapse movies using IncuCyte^{TM} imaging system with built-in software (Essen Instruments).

Chapter 3

Derivation and long-term propagation of rat NS cells: overcoming cell dormancy

3.1 Introduction

3.1.1 Rats in neurological research

Rats have been applied in scientific researches for over a century. In 1895, researchers in Clark University (U.S.) established the first population of domestic rats for physiological studies. Over the years, laboratory rats have been used in genetic research, disease modeling, and drug screening. In neuroscience, laboratory rats have proved valuable in psychological studies of learning and other mental processes. For example, a recent study suggested that rats are capable of metacognition, a mental ability previously only found in humans and nonhuman primates (Foote and Crystal, 2007). In addition, since rat brains

provide easier surgical access than mice, the major neural disorder models, in particular neurodegenerative disease and stroke models, have been established in rats. Transplantation of neural stem cells and/or their progeny into these models can be seen as prototypes of potential cell-replacement therapies for human neural disorders. Availability of rat neural stem cells would avoid the need for immunosuppression and ensure full compatibility for growth and trophic factors. In this chapter, I will describe the derivation of rat NS cell lines and subsequent characterization, particularly of mechanisms regulating cell propagation and quiescence.

3.1.2 Rat neural precursor cells in vitro

For many years, researchers have tried to derive rat neural precursor cell lines from the developing and adult CNS. Although progress has been achieved in establishing mouse and human cell lines, the generation of stable rat neural precursor cultures appears to be difficult. Despite the variation of protocols between labs, most rat neural precursor cells exhibit limited proliferative capacity *in vitro*, leading to spontaneous cell quiescence or crisis after 4-10 weeks expansion. For example, Svendsen et al. (1997) showed that EGF-responsive rat neurospheres derived from foetal striatum could only be expanded for up to 28 days, while precursor cells derived from the same area of fetal mouse brain could be expanded *in vitro* for at least 50 days under the same conditions. In their report, rat precursors spontaneously displayed features of differentiation and apoptosis after 3-4 weeks expansion. A combination of EGF and FGF2 acted synergistically on cell growth, but it did not prevent the final senescence and death of the rat precursors (Svendsen et al., 1997). In monolayer cell cultures, similar observations have also been reported (Kelly et al., 2005).

To date, there is only one rat neural precursor cell population that has been reported to retain long-term proliferation *in vitro.* In 1995, a group of researchers described the

derivation of rat neural precursor cultures derived from the adult hippocampal area (Gage et al., 1995). In their studies, adult rat neural precursor cells were cultured on poly-ornithine and laminin substrates at a high cell density in conditioned medium supplemented with FGF2. Under these conditions, rat neural precursors could propagate for more than 18 months, generating 33 passages in 1.5 years (Palmer et al., 1997). Upon exposure to serum and retinoic acid or forskolin, these cultures were able to generate both neurons and glia cells (Palmer et al., 1997). The same group later reported that an autocrine/paracrine cofactor, glycosylated form of cystatin C (CCg), is a key factor in conditioned medium that promotes the proliferation of rat neural precursor cells (Taupin et al., 2000). However, this observation has yet to be substantiated by additional evidence from other labs. Also, since most of the above observations were obtained from heterogeneous cell populations (Gage et al., 1995; Palmer et al., 1997), the identity of expanded precursor cells remains controversial, particularly when clonal cell lines from these rat precursors were identified to be karyotypically abnormal. Some conflicting results were also observed by others researchers showing adult hippocampus may not contain true neural stem cells (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). Multipotent stem cell populations derived from adult hippocampal area may come from sub from subependyma of the ventricles (Seaberg and van der Kooy, 2002).

The significant difference of proliferative capacity between rat and mouse/human neural precursors *in vitro* raises the question why rat cells spontaneously cease division. One possibility is that rat neural precursors have fundamental difference from mouse and human cells. Alternatively, cultured rat neural precursors may reflect some properties of neural stem cells *in vivo*, which in principle are slow dividing or quiescent (Morshead et al., 1994). If this is the case, the investigation of signals regulating *in vivo* neural stem cells may reveal possible culture conditions that would sustain the proliferation of rat neural precursor cells *in vitro*.

3.1.3 Research Aims

In Chapter 2, I have described the derivation and characterization of human NS cell lines. Here, I will explore the possibility of establishing adherent rat foetal NS cell lines. Previous studies have indicated a limited proliferative capacity of rat neural precursor cells *in vitro* (see above). Therefore the main question to answer in this part of study is how to achieve the long-term stability of cultured rat neural precursor cells. Since EGF, FGF, BMP, and Wnt signals all display regulatory effects on neural precursor cells proliferation and differentiation (see Chapter 1), I examined whether they are involved in the spontaneous quiescence and differentiation of rat neural progenitors *in vitro*.

3.2 Results

3.2.1 Primary cultures of rat foetal neural precursor cells

Rats from two different strains, Fischer 344 and CD (AKA Sprague Dawley), were used to derive rat NS cells. For a better comparison between rat and human/mouse NS cells, I first tried to derive rat NS cells from foetal neural tissue. Rat embryos at embryonic day 13.5 were sacrificed. Foetal brain and spinal cord tissue samples were dissociated into single cells separately and plated onto laminin coated dishes in expansion medium. Initially, the culture conditions that I applied in rat cell cultures were same as those described for mouse NS cell expansion (Conti et al., 2005). Under these conditions, primary rat cells attached within 24 hours after plating, and morphological precursor cells started to divide soon after (Fig 3.1 a, b). During the first 2-3 weeks, the primary rat cell cultures were heterogeneous, containing both Nestin expressing progenitors and Tuj1 positive neuronal cells (data not shown). To enrich for undifferentiated neural precursor cells, primary cultures were transferred onto gelatin coated dishes to eliminate neurons and neuronal

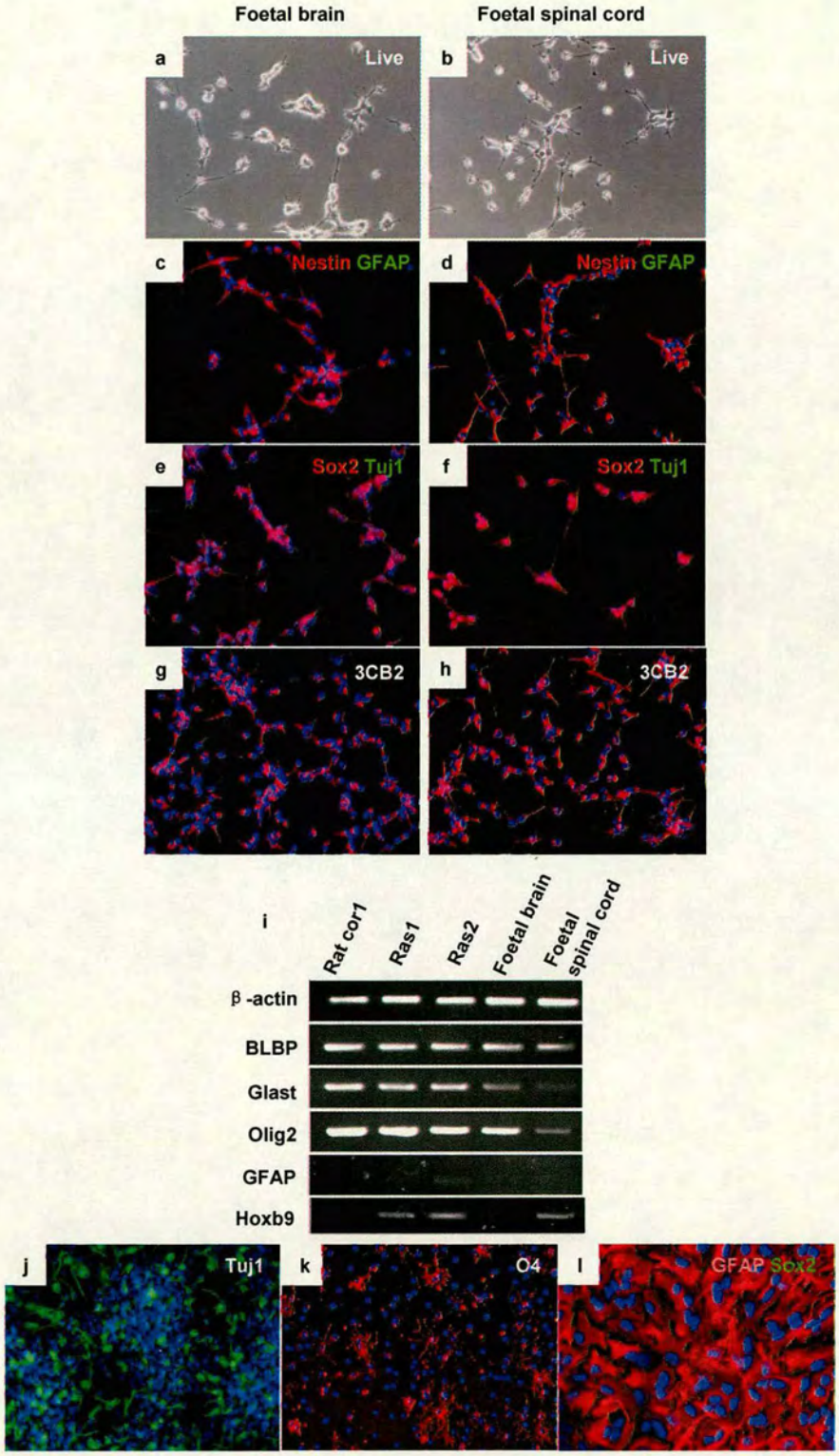

Foetal brain
Foetal spinal cord
a
Live
b
Live
c
Nestin GFAP
d
Nestin GFAP
e
Sox2 Tuj1
f
Sox2 Tuj1
g
3CB2
h
3CB2
i
Rat cor1
Ras1
Ras2
Foetal brain
Foetal spinal cord
β -actin
BLBP
Glast
Olig2
GFAP
Hoxb9
j
Tuj1
k
O4
l
GFAP Sox2

Figure 3.1 Primary rat neural precursor cells

Rat neural precursor cells could be derived from foetal cortex and spinal cord (a, b). After ~3 weeks expansion, primary cultures became morphologically homogeneous and expressed a range of neural precursor markers including Nestin, Sox2, and 3CB2 (c-h), but they did not express GFAP or Tuj1 (c-f). RT-PCR indicated that these cells also expressed BLBP, Glast, and Olig2, as well as regional markers such as Hoxb9 (spinal cord cells) (i). Primary cultures were able to generate Tuj1+ neurons (j), O4+ oligodendrocytes (k), and pure GFAP+/Sox2- astrocytes (l).

progenitors. Similar to mouse NS cells and in contract to human NS cells, the propagation of rat neural precursor cells does not rely on laminin coating. Viable rat cells sustained rapid proliferation on gelatine substrates, displaying a doubling time of 24-48 hours. During this period, the culture medium was renewed by 100% every 2 days, and cells were split at 1:3 once cultures became confluent. Three weeks after (initial) plating, immunostaining indicated that the primary rat cultures were homogeneously Nestin positive and Tuj1 negative (Fig. 3.1 c-f). Cultures at this stage were considered to be passage one rat neural precursor cells. Using the above culture procedures, rat neural precursor populations could be derived from independent foetal brain and spinal cord samples. To date, I have derived 5 independent cultures, three from foetal cortex named Ratcor1, 2, 3, and two from spinal cord named Ras1 and Ras2.

Primary rat neural precursors look similar to mouse NS cells. In the presence of EGF and FGF2, they exhibit bipolar morphology and constant proliferation for up to 2 months (see below). Time-lapse videomicroscopy indicated that they were highly motile and displayed interkinetic nuclear migration (data not shown). When rat neural precursors were plated at clonal density, they generated colonies, but the efficiency (4.7%, 9 colonies from 192 deposited single cells) was lower than mouse or human NS cells (~10%), and the colonies were small. Immunostaining and RT-PCR showed that rat neural precursor cells expressed a set of neural progenitor/radial glia markers including Nestin, Sox2, 3CB2, BLBP, Glast, Olig2, and Vimentin (Fig. 3.1 c-i). Under expansion conditions, primary rat neural precursor cells did not express Tuj1 or GFAP (Fig. 3.1 c-f, i).

Primary rat neural precursor cultures are able to generate both neurons and glial cells. Based on protocols previously developed for mouse NS cells (Conti et al, 2005; Glaser et al., 2007), there were approximately 38.6±4.3% and 21.6±3.2% of cells expressed Tuj1 or O4 respectively at the end of differentiation (based no analyzing 2057 cells from three

independent experiments) (Fig.3.1j, k). Rat neural precursor cells generated pure astrocyte population upon exposure to BMP4 or serum (Fig.3.1l). Rat astrocytes express GFAP, and do not express Nestin or Sox2 (Fig.3.1l). The three lineages derivatives could be generated from individual colonies raised from cultures plated at clonal density. This observation demonstrates that the primary cultures contained proliferative and tripotent neural precursor cells.

3.2.2 Continuous propagation of rat neural precursor cells requires conditioned medium

Unlike mouse or human NS cells that could be continuously expanded *in vitro*, rat neural precursor cells spontaneously reduced cell proliferation and developed stellate morphology after ~2 months expansion (passage ~10) (Fig. 3.2a and Video 3.1). BrdU incorporation and flow cytometry indicated that few cells remained in cycle. The majority of cells (94.6% in average) were arrested at cell cycle G0/G1 phase (Fig. 3.2d and data not shown), significantly different from the proliferative population at early passages (Fig. 3.2e). I observed that all 5 independent rat neural precursor cultures displayed restricted proliferation capacity and generated stellate cells after ~2 months expansion.

The morphological changes and cell cycle arrest imply that rat foetal neural precursor cells may have spontaneously differentiated into cells of other lineage(s). Indeed, immunostaining showed that stellate cells expressed GFAP (Fig. 3.2b), a marker indicating astroglial lineage. However, unlike astrocytes that are generated from rat neural precursor cells upon serum or BMP exposure, stellate cells retained the expression of neural precursor markers such as Nestin, Sox2, and Vimentin (Fig. 3.2c). Stellate cells were viable for at least two months. Continued expression of neural precursor markers requires expansion medium containing EGF and FGF2. When EGF and FGF2 are withdrawn, stellate cells largely differentiated into GFAP-positive and

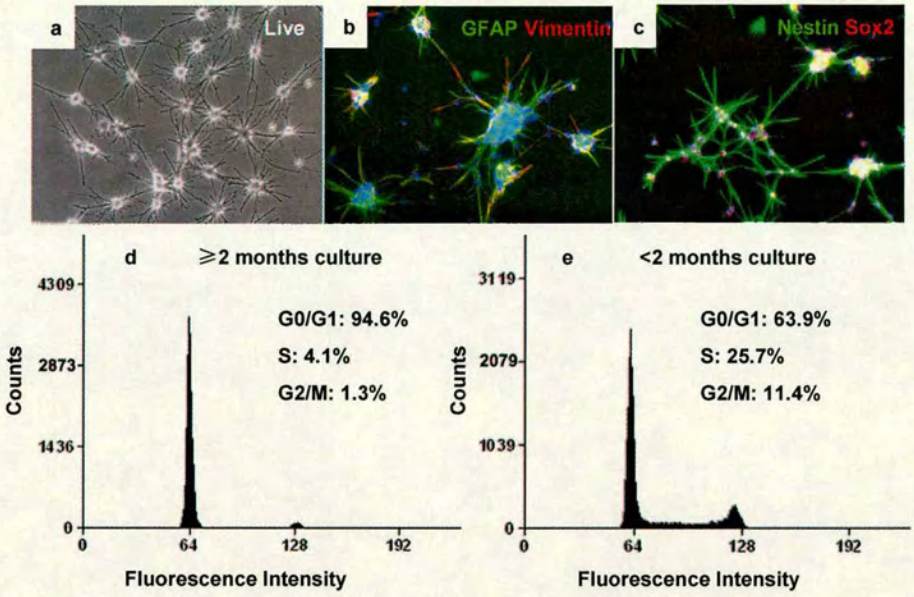


Figure 3.2 Rat neural precursor cells enter dormancy

After ~2 months of initial plating, rat neural precursor cells spontaneously reduced proliferation and developed stellate morphology (a). Immunostaining indicated stellate cells expressed astroglial marker GFAP, but they retained Nestin, Vimentin, and Sox2 expression (b, c). PI staining and flow cytometry confirmed the cell cycle exit of stellate cells by showing 94.6% of cells in average were arrested at G0/G1(d). A control analysis of proliferative cells is illustrated in Fig.3.3e.

Nestin/Sox2-negative astrocytes accompanied by extensive cell death.

The spontaneous ceasing of division is consistent with the earlier observations that rat foetal neural precursor cells have limited proliferative capacity *in vitro* (Svendsen et al., 1997; Kelly et al., 2005). Since long-term expansion was achieved in adult rat neural precursor cultures using conditioned medium (Gage et al., 1995; Palmer et al., 1997), I tested whether conditioned medium could maintain the proliferation of foetal cells. Interestingly, when rat foetal neural precursor cells were cultured at a relative high density (50-80% confluence) and the medium was renewed by 50% every three days, the generation of stellate GFAP expressing cells could be suppressed (Video 3.2). Instead, foetal precursor cells displayed stable proliferation and retained Nestin/Sox2 expression. The continuous division of rat foetal neural precursor cells is fully dependent on exposure to conditioned medium. When conditioned medium was withdrawn, rat cells spontaneously reduced cell division within 2 weeks and develop stellate morphology. Furthermore, when growth arrested GFAP-positive stellate cells were exposed to conditioned medium from proliferative cultures (passage number does not matter), they could re-develop bipolar morphology and re-enter the cycle.

Thus, it seems that adherent rat neural precursor cells spontaneously enter "dormancy" as stellate cells after ~2months *in vitro* expansion, at which stage they upregulate expression of the astroglia marker GFAP. However, the dormant cells are not terminally differentiated as they retain the expression of neural precursor markers such as Nestin and Sox2 and are able to re-enter cell cycle in the presence of conditioned medium. Therefore, the stellate GFAP expressing cells can be regarded as quiescent neural precursor cells, and conditioned medium sustains continuous propagation by overriding cell quiescence.

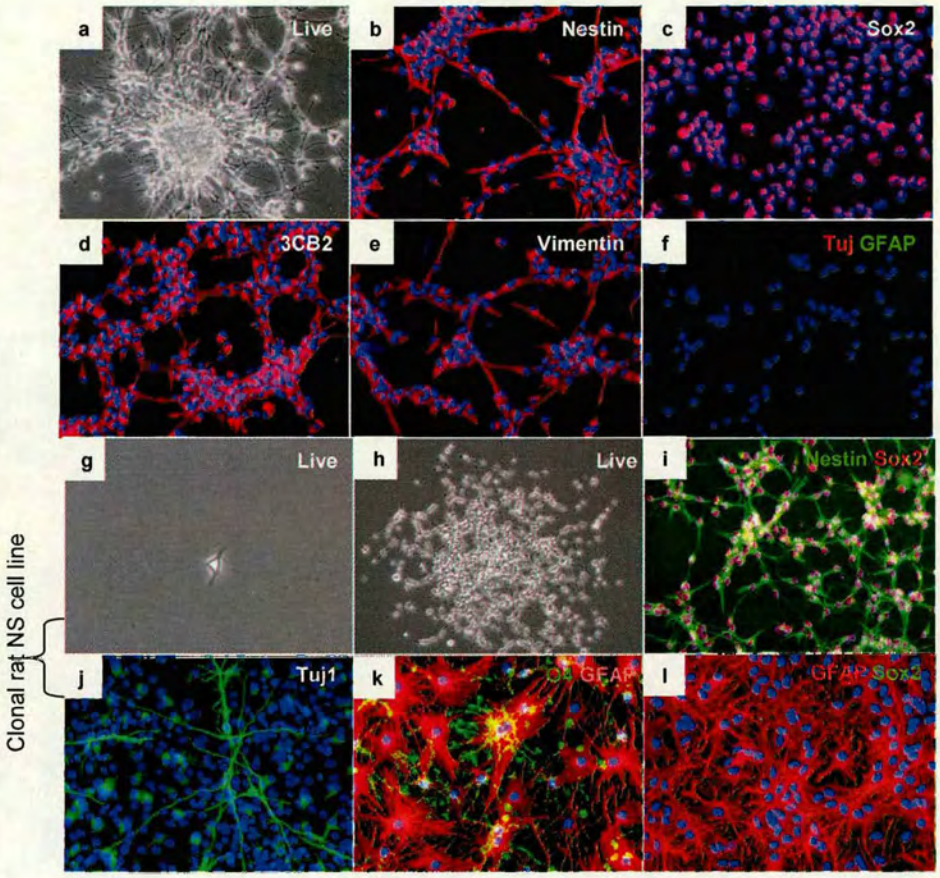
3.2.3 Conditioned medium maintains tripotent and clonogenic rat NS cell lines

In conditioned medium, neural precursor cells derived from rat foetal brain and spinal cord retain bipolar morphology and express a set of neural stem cell/radial glia markers including Nestin, Sox2, 3CB2, Vimentin, Olig2, and BLBP (Fig. 3.3 a-e, m). Tuj1 and GFAP expression could not be detected in expansion cultures (Fig. 3.3f, m). Morphologically, brain and spinal cord cells are indistinguishable, but brain cells divide slower (doubling time 48-72 hours) than spinal cord precursors (doubling time ~24 hours) (Fig. 3.3n). To date, the precursor cells derived from rat foetal brain and spinal cord have been expanded to passage>25 (over 75 generations) and passage >50 (over 150 generations) respectively (Video 3.2 and data not shown).

The precursor cells expanded in conditioned medium are clonogenic. When single brain or spinal cord cells were seeded into each well of 96-well plate by FACS deposition, 8.33% of them generated colonies (16 colonies from 192 deposited single cells) that could be further expanded to establish clonal cell liens (Fig. 3.3 g-i). Neurons, oligodendrocytes, and astrocytes could be derived form clonal cell lines using the differentiation conditions described above (Fig. 3.3 j-l).

In addition, cultured rat neural precursor cells are amenable to genetic modification. For example, when Ras2 cells were transfected with linearized pCAGGFPIP plasmid DNA by Nucleofection, ~30% of them displayed Green Fluorescent Protein (GFP) expression 30 hours after transfection. Transfected cells were placed under selection with puromycin at 2 μg/ml for 6 days, and cells with stable and readily visualised GFP expression were isolated by repeated cell sorting and expanded for more than 2 months (Fig. 3.3 o, p).

These observations indicate that rat foetal neural precursor cells can be maintained as undifferentiated, clonogenic, and tripotent cell lines that are amenable to genetic modification. These cells exhibit the properties to be recognized as neural stem cells *in*


a
Live
b
Nestin
c
Sox2
d
3CB2
e
Vimentin
f
Tuj GFAP
g
Live
h
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Nestin Sox2
Clonal rat NS cell line
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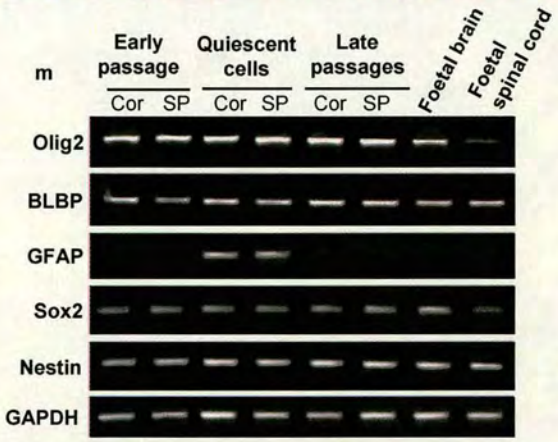
O4 GFAP
l
GFAP Sox2


m
Early passage
Quiescent cells
Late passages
Foetal brain
Foetal spinal cord
Cor SP Cor SP Cor SP
Olig2
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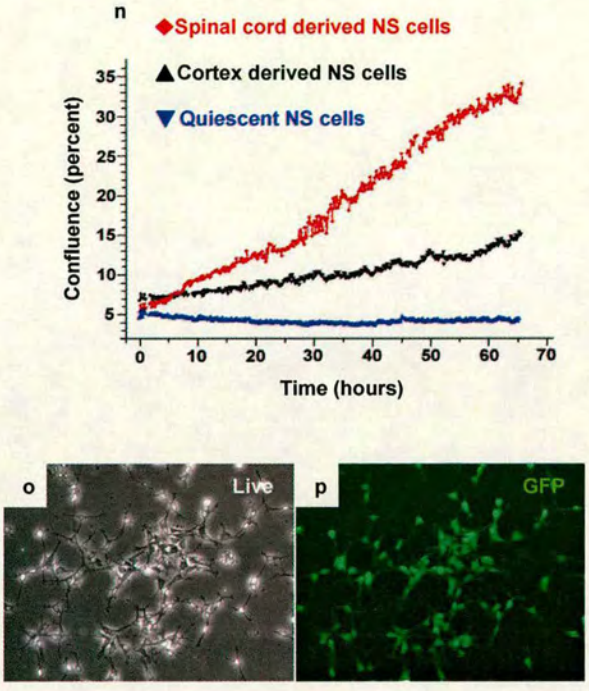


Figure 3.3 Conditioned medium maintains tri-potent and clonogenic rat NS cell lines

In conditioned medium, rat NS cells retain continuous proliferation without GFAP or Tuj expression (a). Brain and spinal cord NS cells at late passage exhibit the same gene expression pattern as cells at early passages (b-f, m). Clonal and tripotent cell lines can be derived from single cells in conditioned medium (g-i). Rat cortex derived NS cells show slower division than spinal cord cells (n). Rat NS cells are amendable to genetic modification. Ras-2 NS cells was transfected with pCAGGFPIP plasmid DNA to induced GFP expression (o, p).

vitro. The long-term propagation of rat foetal NS cell lines is dependent on conditioned medium, unlike mouse and human NS cells.

3.2.4 Cystatin C, Wnt, and PDGF signals do not overcome rat NS cell quiescence

Since conditioned medium overcomes spontaneous cell quiescence of both rat foetal NS cells and adult hippocampal progenitor cells, one may speculate that there is an auto/paracrine factor(s) other than EGF and FGF2 that stimulate the cell proliferation. In 2000, Taupin et al. reported that a glycosylated form of cystatin C (CCg) could be purified from conditioned medium of adult rat hippocampal cell cultures, and CCg in combination with FGF2 promoted the proliferation of rat neural precursors both *in vitro* and *in vivo* (Taupin et al., 2000). However, no additional evidence has been reported to support this observation. In rat NS cell cultures, although CCg mRNA is expressed by both proliferative and quiescent rat NS cells (Fig 3.4a), fresh expansion medium (renewed by 100% change daily) supplemented with CCg could not replace the conditioned medium to overcome cell quiescence (data not shown).

Wnt proteins have recently been recognized as stem cell growth factors (Willert et al., 2003), and Wnt signaling is involved in regulation of stem cell proliferation and differentiation (Reya and Clevers, 2005). RT-PCR indicated that rat NS cells expressed mRNA encoding Wnt proteins (Wnt4 and Wnt5a) and Wnt receptors (Fzd1, 4, 9) (Fig 3.4a), suggesting Wnt signals may regulate NS cell proliferation. However, immunostaining indicated that β-catenin was not detected in nucleus of either proliferative NS cells or quiescent stellate cells (Fig. 3.4b and data not shown) (see Chapter 1 for details of Wnt signaling). Application of TWS119, a glycogen synthase kinase-3β (GSK-3β) inhibitor (Ding et al., 2003), stimulated nuclear translocation of β-catenin (Fig. 3.4c), but did not lead to continuous proliferation. Instead, a small number of rat NS cells underwent neuronal differentiation after exposure to TWS119, displaying Tuj1 expression

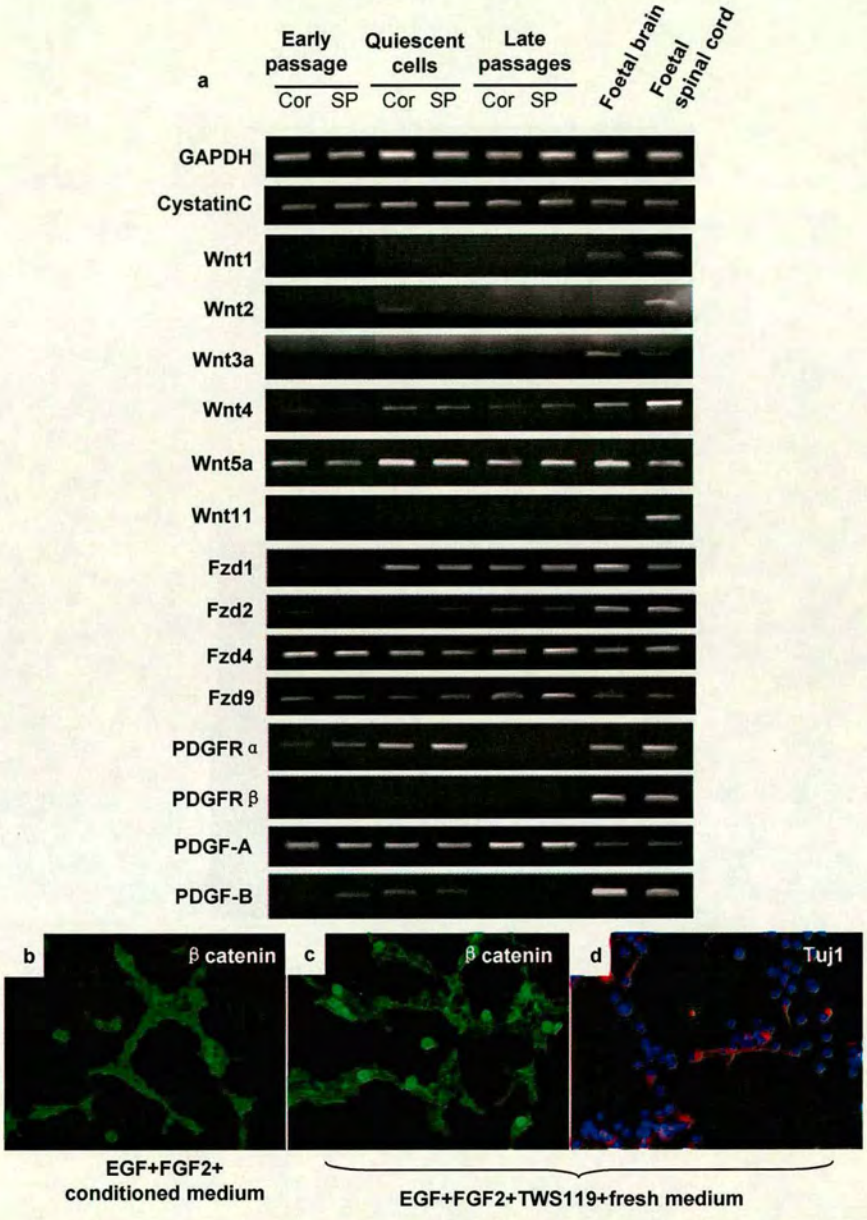


Figure 3.4 CCg, Wnt, and PDGF signals in rat NS cells

RT-PCR indicates Rat NS cells express CCg, Wnts, PDGF, and relative receptors (a). Proliferative rat NS cells do not display β -catenin translocation into the nucleus (b). GSK3 inhibitor TWS119 artificially activated canonical Wnt pathway (c), but it led to neuronal differentiation instead of overcoming cell quiescence (d).

even in the presence of EGF and FGF2 (Fig. 3.4d). To examine the possibility that Wnt proteins may act through non-canonical pathways, I applied Wnt proteins (Wnt3a or Wnt5a) in fresh expansion medium. However, rat NS cells exposed to exogenous Wnt proteins still spontaneously entered dormancy within 3 weeks after conditioned medium withdrawal, at the same time when a parallelly cultured rat NS cell population ceased proliferation in the absence of conditioned medium (data not shown). In addition, application of Wnt receptor inhibitors (Dickkopf or Secreted Frizzled Related Protein) in conditioned medium did not lead to cell quiescence or reduced cell proliferation (data not shown). These observations suggest that Wnt signals do not contribute to the essential culture environments for rat NS cell self-renewal.

In addition to CCg and Wnts, rat NS cells expressed PDGF-A and PDGF receptor α (Fig. 3.4a). PDGF is classically viewed as a regulator of glial differentiation (Raff et al., 1988; Hart et al., 1989). Recently, it has been shown that PDGFRα is expressed in adult SVZ type B cells, and PDGF stimulates the proliferation of adult mouse neural precursor cells both *in vivo* and *in vitro* (Jackson et al., 2006). However, applying PDGF or PDGF receptor inhibitors AG1295/96 (Banai et al., 1998; Tse et al., 2002) in rat NS cell cultures did not affect either proliferation or spontaneous quiescence (data not shown).

3.2.5 Conditioned medium overcomes cell quiescence by antagonizing BMP signals

The observations that CCg, Wnt, and PDGF signals do not overcome cell quiescence lead to the exploration of other auto/paracrine factors. Since stellate cells express GFAP, a maker for astroglia cells, I speculate that glial-inducing factors such as BMPs could be involved rat NS cell quiescence. Indeed, RT-PCR indicates that rat NS cells express BMP3 (GDF10) and BMP receptors, and both are up-regulated in quiescent cells (Fig 3.5a). In addition, rat NS cells express the BMP antagonist Chordin, which is down-regulated in quiescent cells (Fig 3.5a-c). Thus, it appears that the balance between

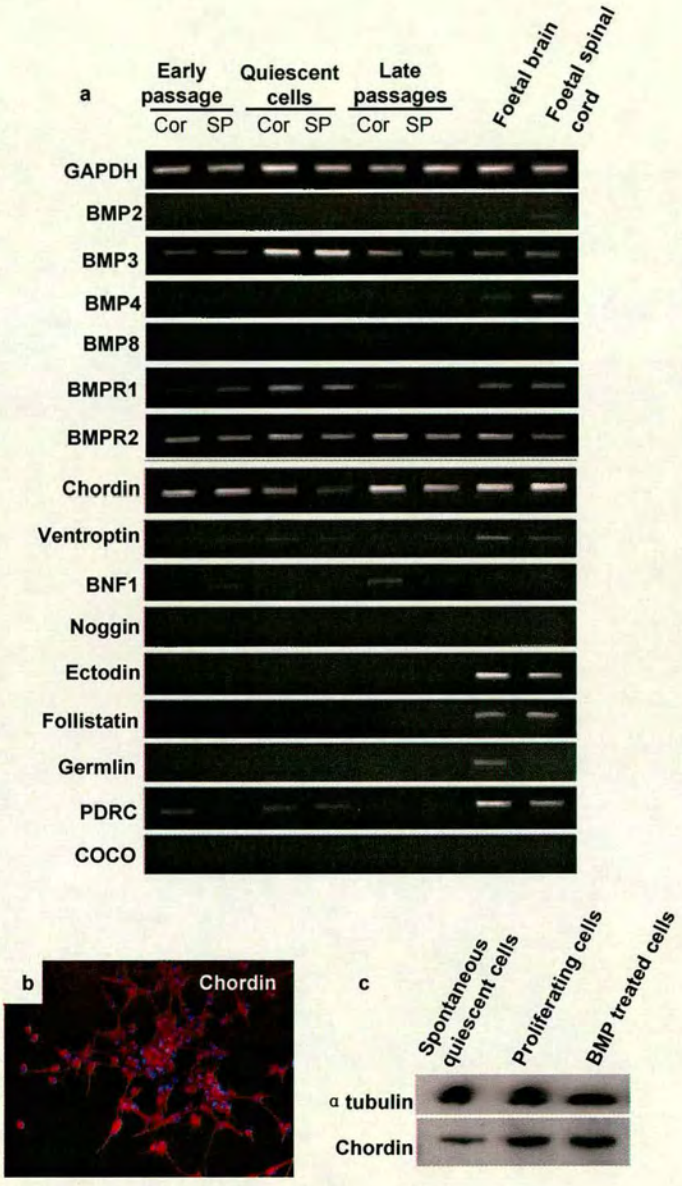


Figure 3.5 Rat NS cells express BMP3 and BMP antagonist Chordin

PR-PCR indicates rat NS cells express BMP3 and BMP receptors, which are up-regulated in quiescent cells (a). Rat NS cells also express BMP antagonists Chordin, which are confirmed by RT-PCR, immunostaining, and Western-blots (a-c).

BMP and BMP antagonistic signals could regulate the propagation and quiescence of rat NS cells. This hypothesis makes three key predictions: 1) BMP signals should induce rat NS cell quiescence; 2) conditioned medium should antagonize BMPs; and 3) a combination of BMP antagonists and fresh expansion medium should replace conditioned medium to overcome spontaneous cell quiescence.

To investigate the effects of BMP proteins on cell quiescence, I plated rat NS cells at low density and renewed culture medium by 100% change daily. Since BMP3 is not readily available, I applied recombinant BMP4 protein in rat NS cell cultures (Ebendal et al., 1998; Kawabata et al., 1998). Previous data has shown that BMP4 alone induces rat NS cells to generate astrocytes that express GFAP but not Nestin or Sox2 (Fig. 3.6 a-c). In the presence of EGF and FGF2, although rat NS cells exposed to BMP4 rapidly ceased division and developed GFAP expression, they retained Nestin/Sox2 expression and displayed stellate morphology. Flow cytometry indicated that the majority of stellate cells were arrested at cell cycle G0/G1 phase (Fig.3.6d-g and Video 3.4), similar to spontaneous cell quiescence. The BMP4 induced cell quiescence could occur at any passage, including primary cells before they undergo spontaneous quiescence (data not shown). When BMP4 is replaced by conditioned medium (from other proliferative cultures), the stellate cells were able to re-enter the cell cycle as tripotent NS cells.

As expected, conditioned medium from proliferative rat NS cells displayed BMP-antagonistic effects. When rat NS cells were treated with conditioned medium and BMP4 at 100ng/ml (10 times more than the dose in astrocyte differentiation), only a small number of cells became GFAP positive (Fig. 3.7a, b). The majority of cells remained in cell division and retained Nestin/Sox2 expression (Fig. 3.7c). When plated at low density, these cells were able to generate small colonies (Fig. 3.7a).

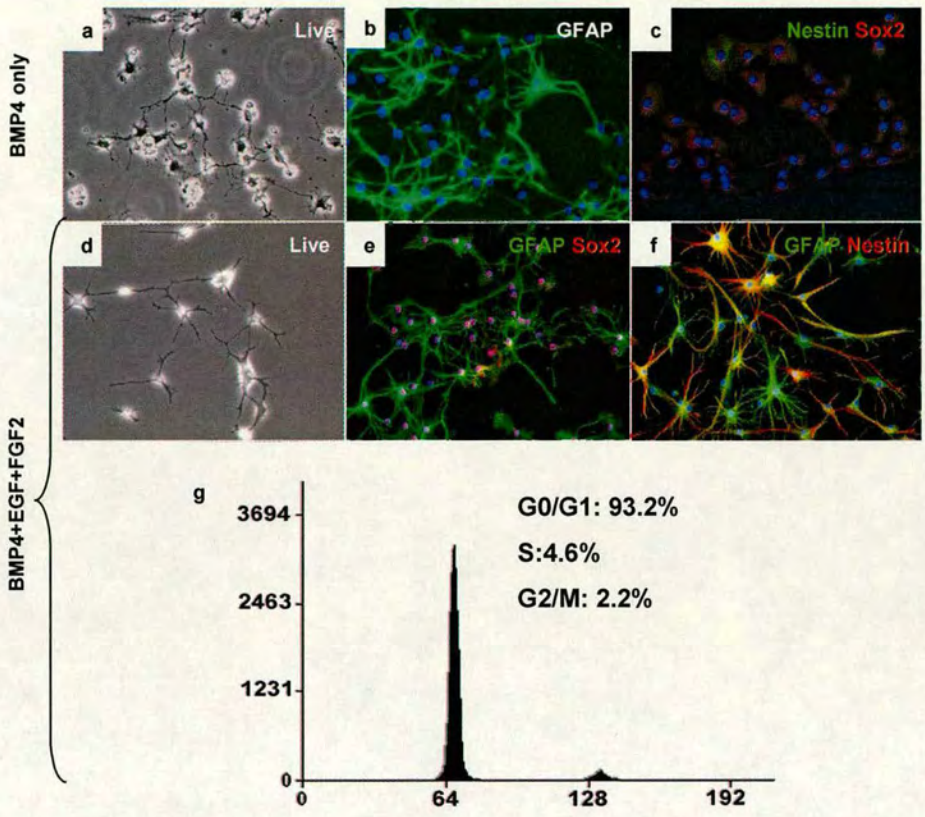


Figure 3.6 BMP signals induce rat NS cell quiescence

BMP4 alone induces rat NS cells differentiation into astrocytes (GFAP+, Nestin-, Sox2-) (a-c). In the presence of EGF and FGF2, BMP4 exposure induces the generation of stellate quiescent cells, expressing GFAP, Nestin, and Sox2. (d-g).

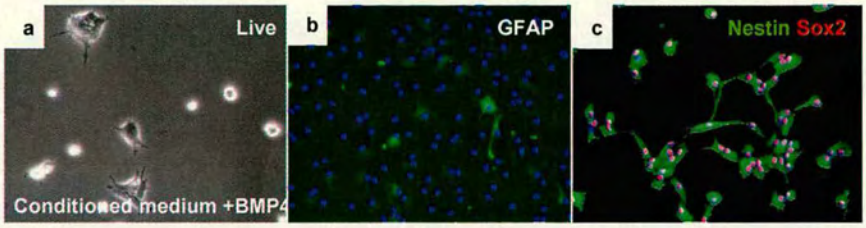


Figure 3.7 Conditioned medium antagonizes BMP signaling

In conditioned medium, BMP4 up to 100ng/ml did not lead to cell quiescence. Rat NS cells under this condition retained proliferation and generated colonies at low density (a). Immunostaining indicated that BMP-induced GFAP expression was suppressed in conditioned medium (b), and all cell retained Nestin and Sox2 expression (c).

Lastly, I tested whether a combination of BMP antagonists and fresh expansion medium would replace conditioned medium to propagate rat NS cells. Since recombinant Chordin does not have consistent biological activity, I applied the alternative BMP antagonist Noggin (Smith and Harland, 1992) to rat NS cell expansion cultures. Indeed, rat NS cells exposed to Noggin bypassed the spontaneous quiescence without recourse to conditioned medium, and retained stable proliferation for at least 15 passages (over 45 generations) (Fig. 3.8a). Under these conditions, proliferative NS cells homogeneously express Nestin and Sox2 but not GFAP (Fig. 3.8b, c), and they are able to generate clonal NS cell lines that display tripotent differentiation potential (Fig 3.8 d-f). In addition, Noggin in fresh medium could also activate quiescent stellate cells, allowing them to re-enter cell cycle and to re-establish proliferative cell populations (Video 3.3).

Thus, the above three experiments demonstrate that conditioned medium overcomes rat NS cell quiescence by antagonizing BMP signals.

3.2.6 TGFβ1, Activin-b, and GDF11 do not induce cell quiescence

As mentioned in the Introduction, BMPs belong to the TGFβ family that includes structurally and functionally related members such as TGFs, GDFs, and Activins. RT-PCR indicates that, in addition to BMP3 and BMP receptors, rat NS cells also express TGFβ1/2, Activin-a/b, GDF11/15, and their receptors (Fig. 3.9a). To investigate whether TGFβ/Activin/GDF signals also contribute to the NS cells quiescence, I applied TGFβ1, Activin-b, and GDF11 proteins in cultures either individually or in combination. Although in a control culture TGFβ1/Activin-b/GDF11 induced haemoglobin expression in K562 cells (data not shown), these proteins did not lead to cell cycle arrest of rat NS cells. Instead, rat NS cells exposed to TGFβ1/Activin-b/GDF11 retained proliferation and homogeneous Nestin/Sox2 expression for at least 7 days, and GFAP expression was not detected (Fig. 3.9 b-j and Video 3.5, 3.6, 3.7).

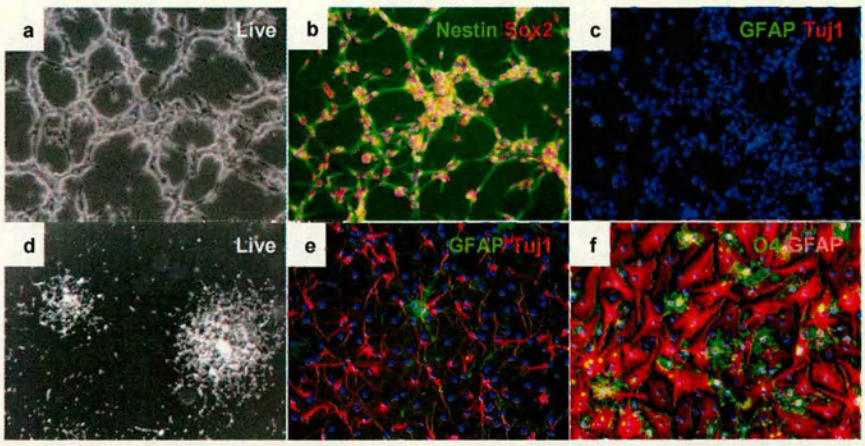


Figure 3.8 Noggin in expansion medium maintains clonogenic rat NS cell lines

In fresh expansion medium, cell quiescence and differentiation are efficiently suppressed by addition of Noggin (a-c). Noggin together with EGF/FGF2 can replace conditioned medium to generate clonal and tri-potent rat NS cell lines (d-f).

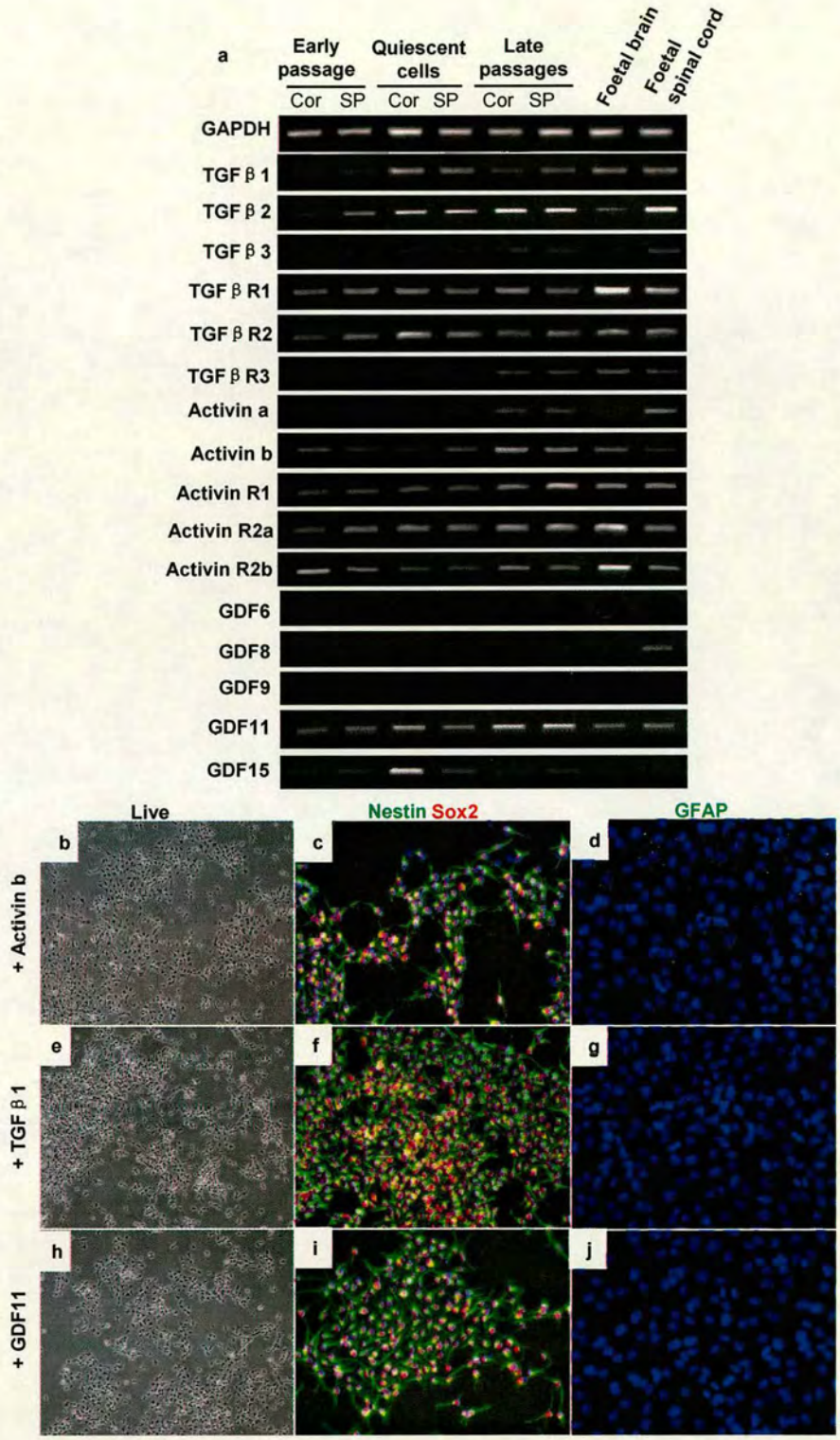

a
Early passage
Quiescent cells
Late passages
Cor SP Cor SP Cor SP
Foetal brain
Foetal spinal cord
GAPDH
TGF β 1
TGF β 2
TGF β 3
TGF β R1
TGF β R2
TGF β R3
Activin a
Activin b
Activin R1
Activin R2a
Activin R2b
GDF6
GDF8
GDF9
GDF11
GDF15
Live
Nestin Sox2
GFAP
b
c
d
+ Activin b
e
f
g
+ TGF β 1
h
i
j
+ GDF11

Figure 3.9 TGF β , Activin, and GDF do not induce cell quiescence

RT-PCR indicates that rat NS cells and/or quiescent cells express Activin a/b, TGF β 1/2, GDF11/15, and their receptors (a). However, addition of Activin b, TGF β 1, and GDF11 into fresh expansion medium did not lead to cell quiescence. Rat NS cells under these conditions retained proliferation and Nestin/Sox2 expression without developing GFAP expression (b-j).

3.2.7 BMP signaling induces cell quiescence via Smad cascades

Having established that BMP signals are responsible for NS cell quiescence, I examined through which signaling pathway BMP proteins exert their effects, specifically the involvement of the BMP-Smad cascade and the BMP-MAPK cascade (see Chapter 1 for details of BMP signaling pathway). Immunostaining indicated that both spontaneous and BMP4-induced quiescent cells displayed phosphorylation of Smad1/5/8 (Fig 3.10a, b), while Smad1/5/8 phosphorylation could hardly be observed in proliferative rat spinal cord NS cells except those in mitotic prophase (possibly due to cross-talking with other signaling pathways or nonspecific antibody staining) (Fig 3.10d). Proliferative rat foetal brain NS cells exhibit low level of Smad1/5/8 phosphorylation. Their slow division compared with spinal cord NS cells might result from inefficient inhibition of BMP-Smad signaling (Fig. 3.10c). Western blot analyses showed the total level of Smad1/2/3/5/8 was slightly increased in quiescent cells (Fig. 3.10e). Along with the phosphorylation of Smad 1/5/8, phosphorylation of p38 was also observed in BMP4-induced quiescent cells (Fig. 10f). However, inhibition of p38 phosphorylation by MAP kinase inhibitors SB203580 and PD169316 could not overcome NS cell quiescence (in the presence of EGF/FGF2) or block their differentiation into astrocytes (without EGF/FGF2) (Fig. 3.10 g-j). These observations suggest that BMPs induce cell quiescence through the canonical BMP-Smad cascade. Although BMP-MAPK signaling is also activated under culture conditions, it is not necessary for either NS cell quiescence or astrocyte generation.

3.2.8 FGF2, not EGF, blocks astrocyte differentiation response to BMP

In the above experiments, the quiescence of rat NS cells occurs in the presence of EGF and FGF2. When EGF and FGF2 are withdrawn, BMP alone induces the generation of astrocytes from rat NS cells (Fig. 3.6a-f and Fig. 3.11a-c). Thus, the question arises whether EGF and FGF2 are both required for NS cells during dormancy. To answer this

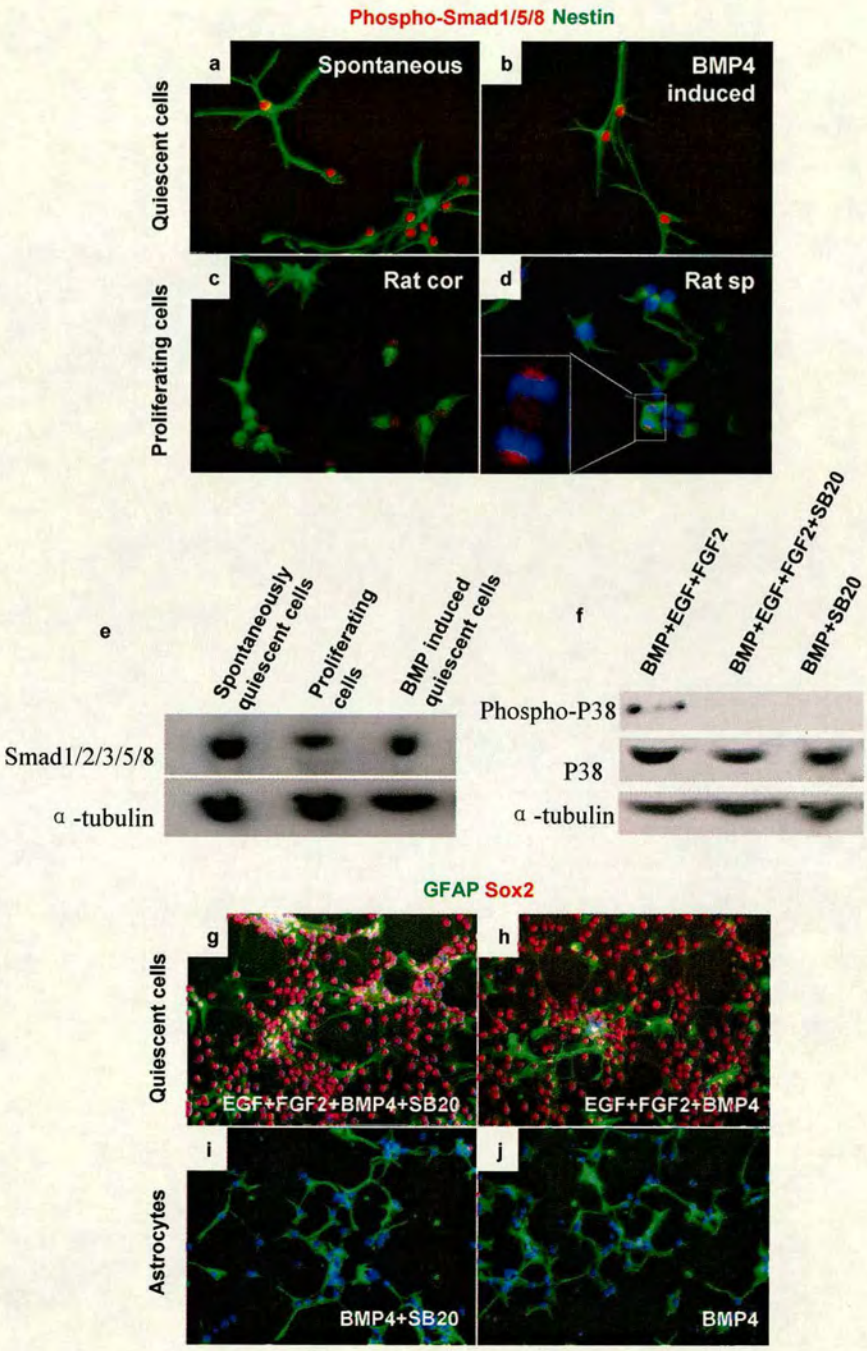

Phospho-Smad1/5/8 Nestin
a
Spontaneous
b
BMP4 induced
Quiescent cells
c
Rat cor
d
Rat sp
Proliferating cells
e
Spontaneously quiescent cells
Proliferating cells
BMP induced quiescent cells
Smad1/2/3/5/8
α -tubulin
f
BMP+EGF+FGF2
BMP+EGF+FGF2+SB20
BMP+SB20
Phospho-P38
P38
α -tubulin
GFAP Sox2
g
h
Quiescent cells
EGF+FGF2+BMP4+SB20
EGF+FGF2+BMP4
i
j
Astrocytes
BMP4+SB20
BMP4

Figure 3.10 BMPs induce cell quiescence via Smad cascades

Both spontaneous and BMP4-induced quiescent cells exhibit Smad1/5/8 phosphorylation (a, b). Slow dividing rat brain NS cells show a low level of Smad1/5/8 phosphorylation (c), which cannot be detected in proliferating spinal cord cells except those in mitosis phase (d). Western bolts show that the total level of Smad1/2/3/5/8 slightly increases in quiescent cells (e). Applying P38 inhibitor SB203580 does not inhibit BMP4 induced cell quiescence or astrocyte differentiation (f-j).

question, I cultured rat NS cells in fresh medium supplemented with EGF or FGF2 only together with 10ng/ml BMP4. In cultures supplemented with EGF, rat NS cells rapidly down-regulated Nestin/Sox2 expression and differentiated into GFAP expressing astrocytes (Fig. 3.11 d-f). In contrast, in cultures with FGF2, the generation of astrocytes was largely inhibited. Although GFAP expression was induced and cells entered dormancy after BMP4 exposure, the majority of cells retained Nestin/Sox2 expression and developed stellate morphology, a phenotype similar to quiescent cells (Fig. 3.11 g-i). When BMP4 is withdrawn, the majority of cells re-entered cell cycle in the presence of conditioned medium (with EGF and FGF2). Therefore, it appears that, in the presence of BMP signals, FGF2 plays a more critical role than EGF in maintaining Nestin/Sox2 expression in rat NS cells.

3.2.9 Noggin promotes cell proliferation of adult rat SVZ neural precursors

The above data has demonstrated that NS cells derived from rat foetal brain and spinal cord can be extensively expanded using conditioned medium or Noggin to avoid cell quiescence. Since the adult brain is also an importance source for derivation of neural precursor cells *in vitro*, I wondered whether such culture conditions could be applied in adult cell cultures.

To derive adult rat neural precursor cells, I dissected the lateral wall of adult SVZ and dissociated tissues into single cells. After being plated in expansion fresh medium supplemented with EGF and FGF2, adult precursor cells underwent transient proliferation for 2-3 weeks before they ceased division and generated stellate cells (Fig. 3.12 a-c and Video 3.8). Unlike foetal NS cells, conditioned medium of adult SVZ precursor cells did not overcome the spontaneous cell quiescence (data not shown). Nevertheless, applying Noggin could maintain the proliferation of adult rat neural precursors for at least 2 months (Fig. 3.12d and Video 3.9). Proliferative cells expanded with Noggin expressed Nestin and

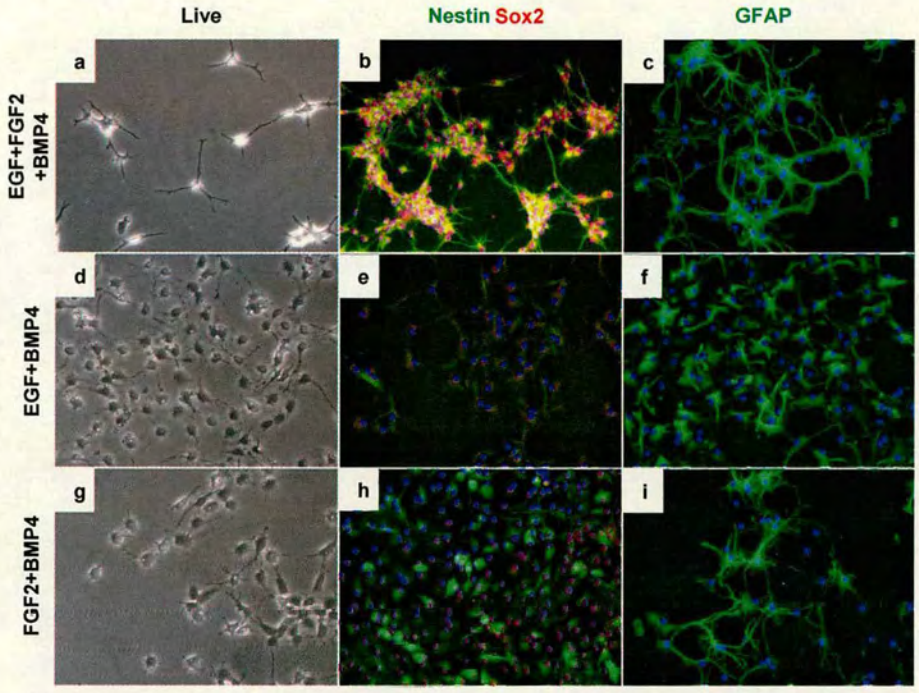


Figure 3.11 FGF2 retains Nestin/Sox2 expression in quiescent NS cells

In the presence of EGF and FGF2, BMP4 induces cell quiescence (a-c). NS cells cultured with EGF and BMP4 rapidly differentiated into astrocytes (d-f). Most rat NS cells cultured with FGF2 and BMP4 retained Nestin/Sox2 expression (g, h), while the majority of cells also developed GFAP expression (i).

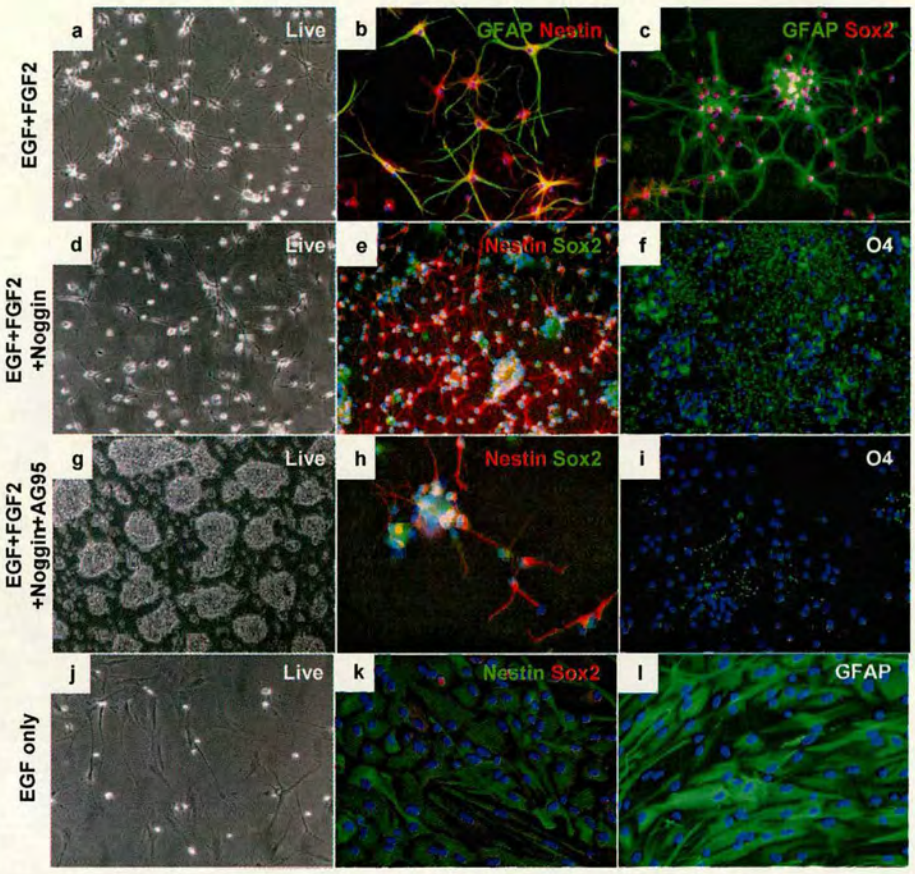


Figure 3.12 Neural precursors derived from adult rat SVZ

Neural precursor cells could be derived from adult rat SVZ. SVZ precursor cells displayed limited proliferative capacity in medium containing EGF and FGF2 (a-c). Extended proliferation could be achieved by addition of Noggin into expansion medium, although ~20% of cells spontaneously differentiated into oligodendrocytes (d-f). PDGF receptor inhibitor AG1295 could partly inhibit the generation of oligodendrocytes, but cultures became loosely attached (g-i). Adult rat neural precursor cells differentiated into astrocytes in EGF only cultures (j-l).

Sox2, and they did not express GFAP (Fig. 3.12e and data not shown). However, cultures under this condition were not homogeneous. Approximately 23.4% of adult precursor cells spontaneously differentiated into O4 positive oligodendrocytes (Fig. 3.12f). The oligodendrocyte generation could be partly inhibited by applying PDGF receptor inhibitors AG1295 or AG1296 in cultures, but these inhibitors led to loose attachment and formation of cell aggregates (Fig 3.12 g-i). Adult SVZ neural progenitors were neurogenic *in vitro*. Under differentiation conditions, they were able to generate neurons (Tuj1+) and astrocytes (GFAP+, Nestin-, Sox2-) (data not shown).

The observation that FGF2 is more important than EGF in maintaining Nestin and Sox2 expression is also mirrored in adult neural precursor cultures. In cultures with EGF only, the majority of cells spontaneously differentiated into astrocytes, displaying flat morphology, GFAP expression, and down-regulating Nestin and Sox2 expression (Fig. 3.12 j-l and Video 3.10). Noggin and EGF together could maintain the propagation of adult SVZ precursor cells (Video 3.11), but the spontaneous oligodendrocyte generation remained (data not shown).

3.3 Discussion

In this chapter, I demonstrated the long-term propagation of rat foetal NS cell lines by overcoming cell quiescence. The quiescence of rat NS cells is induced by BMP signaling through the Smad cascade. This can be prevented by conditioned medium or BMP antagonists (Fig. 3.13). Thus, the balance between auto/paracrine BMP and BMP antagonistic signals play important roles in regulating the proliferation and quiescence of rat NS cells.

I observed that the spontaneous cell cycle arrest consistently occurs in primary cultures at ~2 months after initial plating. One explanation for this phenomenon may be that the primary cultures are heterogeneous. Initially, BMP signals may be produced at low levels or antagonized by factor(s) released from differentiating cells. After the culture is taken over by proliferative NS cells, usually after 1-2 months after initial plating, BMP signals become dominant and induce cell quiescence. Another explanation is that there is an "internal clock" in rat neural precursor cells. In other words, the properties of precursor cells alter in culture conditions in a temporally defined manner after initial plating. In this case, neural precursor cells may develop responsiveness or become more sensitive to BMP signals *in vitro*. However, the observation that exogenous BMP4 could arrest cell cycle before the spontaneous cell quiescence demonstrates BMP responsiveness in primary cultures, providing little support to the second explanation.

Quiescent rat NS cells are an intriguing cell population. On one hand, they hardly divide and they express GFAP, displaying properties of astroglial cells. On the other hand, these cells retain Nestin/Sox2 expression, are able to re-enter cell cycle, and can generate both neurons and glial cells upon BMP4 withdrawal. These observations suggest quiescent NS cells retain properties of stem cells, and therefore I speculate that they represent a transitional cell population between neural stem cells and differentiated astrocytes. In this case, BMP signals trigger the first step of glial differentiation of rat NS cell, resulting in cell cycle arrest and GFAP expression. However, the generation of mature astrocytes cannot be accomplished due the presence of mitogens that retain stem cell potency in quiescent cells (Fig. 3.13).

My data indicate that EGF and FGF2 play different roles in NS cell cultures. Although EGF appears to be the major mitogen for NS cell self-renewal (Pollard et al., 2006; and Chapter 2), it does not inhibit astroglial differentiation induced by BMP signals. In

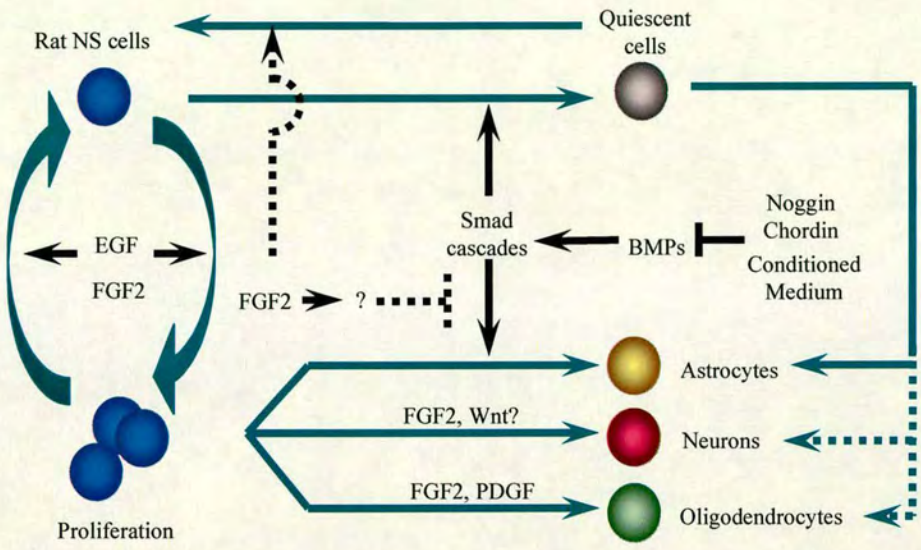


Figure 3.13 Model for *in vitro* rat NS cells

EGF and FGF2 stimulate the proliferation of rat NS cells. FGF2 alone induces neuronal differentiation, and Wnt signals may also favor the generation of neurons. PDGF and FGF2 induce rat NS cells differentiate into oligodendrocytes. BMPs via Smad cascade induce rat NS cells to differentiate into astrocytes. Noggin and Chordin bind to BMP proteins and consequently prevent their activation of BMP receptors. FGF2 also acts antagonistically to BMP signals by inhibiting astrocyte generation, which leads to rat NS cells entering dormancy. Quiescent cells retain potentials to differentiate into astrocytes, but it is not clear whether they are able to directly differentiate into neurons or oligodendrocytes. Quiescent cells are able to re-enter the cell cycle as proliferative and tripotent NS cells, but whether this process requires FGF2 is not clear.

contrast, FGF2 exhibit some level of "anti-BMP" effects by retaining Nestin/Sox2 expression in quiescent cells. Earlier studies have shown that FGFs and BMPs act antagonistically in several organs including the CNS, tooth, and limb (Niswander and Martin, 1993; Neubuser et al., 1997; Lillien and Raphael, 2000). Although it has been suggested that the cross-talk between Erk/MAPK and BMP-Smad cascades may contribute to the above observations (von Bubnoff and Cho, 2001), the detailed mechanisms are not fully understood. More careful experiments are required in future to elucidate the relationship between BMP and FGF signals.

Another issue to consider about the quiescent rat NS cells is whether they have *in vivo* counterparts. Since quiescent cells display a neurogenic and astroglia-like phenotype, they could reflect some properties of adult neural precursors, notably the forebrain SVZ Type B cells that are considered as possible adult neural stem cells. Type B cells display several similarities to quiescent NS cells: 1) they are slow dividing or quiescent cells expressing GFAP and PDGFR-α (Doetsch et al., 1999; Seri et al., 2001; Jackson et al., 2006); 2) they are able to generate proliferative precursor cells that later produce young neurons (Doetsch et al., 1999; Alvarez-Buylla and Garcia-Verdugo, 2002); and 3) they reside in niche that contains BMP, Noggin, and FGF signals (Lim et al., 2000; Doetsch et al., 2002; Zheng et al., 2004). Under this speculation, cultured rat NS cells could serve as a model for adult neural stem cells. However, careful studies are required to compare quiescent cells and adult SVZ Type B cells in more details. For example, transplantation of proliferative or quiescent rat NS cells into adult SVZ would illustrate to what extend they share similarities in behaviour. In addition, it will be interesting to investigate whether quiescent cell populations can be generated from mouse and human NS cells.

The observation described in this chapter that BMP signaling contributes to the dormancy and GFAP expression of rat NS cells largely consists with previous studies showing

BMPs inhibit proliferation of foetal cortex precursors and promote astroglial differentiation (Mabie et al., 1999; Mehler et al., 2000; Hebert et al., 2002). Indeed, when Lim et al. (2000) over-expressed BMP7 in the ependymal cells of adult mouse, they observed a decrease in proliferation that may be responsible for the decrease in regeneration of neuroblasts after AraC and BMP7 treatment. In addition, the antiproliferative role of BMP over-expression has also been demonstrated in glioblastoma cells in the adult brain (Piccirillo et al., 2006). Yet, a more recent study of Colak et al., (2008) suggested that the role of BMP may be more complicated than expected. By conditional deletion of Smad4 or infusion of Noggin in the adult subependymal zone, Colak et al., observed that BMP-mediated signaling is active in adult neural stem cells and is crucial to initiate the neurogeneic lineage and to inhibit oligodendrogliogenesis. While this result seems contradictory to earlier observations, one explanation could be that the bio-function of BMP signal may depend on its level (Colak et al., 2008). In the adult mouse SVZ, where BMPs and BMP antagonists both exist (Lim et al., 2000), BMP signals may be maintained at a low level, showing no effects on cell proliferation but favour neurogenesis. While this balance is changed (e.g. by over-expression of BMPs or under culture conditions), BMP signals may reach a relative high level and thus inhibit cell proliferation and induce astroglia differentiation. In my rat NS cell cultures without conditioned medium or Noggin, BMPs generated by NS cells may exhibit a 'high-level effect', leading to spontaneous cell cycle arrest and astroglial differentiation. When conditioned medium or Noggin are applied, the BMP signals should be adjusted to a low level and thus allow NS cell to proliferate and retain neuronal differentiation potential. However, this hypothesis will not be confirmed unless the levels of BMP signals as well as their effects are carefully measured. Also, it is worthwhile to test whether such phenomenon can be re-produced in human and mouse cell cultures.

3.4 Materials and Methods

3.4.1 Tissue culture materials and reagents

Unless specified, the culture materials and reagents for rat NS cells are same as those in human NS cell cultures, which are described in Materials and Method 2.4.1.

3.4.2 Animals

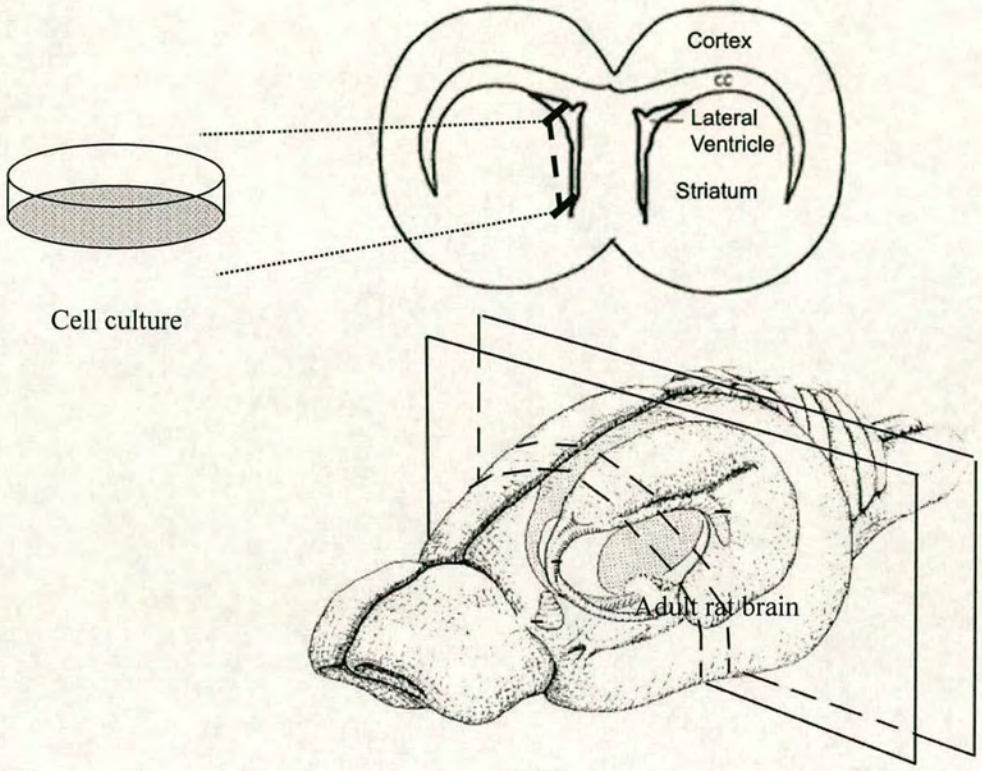
Rat tissues were derived from two different strains, Fischer 344 and CD (AKA Sprague Dawley). Rat embryos were obtained at the University of Edinburgh. Adult rats came from Charles River Laboratories.

3.4.3 Dissection and primary cultures

To derive rat foetal NS cells, rat embryos were sacrificed at embryonic day 13.5. Foetal brain and spinal cord were carefully dissected, and outer membranes were removed. Tissue samples were then incubated with a mix of PBS and Accutase (1:1) at 37°C for 5 minutes and mechanically dissociated into single cells. Cells were plated onto laminin coated T25 flask in expansion medium comprising Euromed-N (Euroclone) or RHB basal medium (StemCellScience), L-glutamine (2mM final, Gibco), modified N2 supplement (Ying and Smith, 2003), B27 (20ml/L final, Invitrogen), Penicillin-Streptomycin (10ml/L final, Sigma), and 10ng/ml of both mouse EGF (Peprotech) and human FGF-2 (Peprotech). Medium was renewed by 50% every two days.

To derive rat neural precursor cells from adult SVZ, adult rats at ~3months old were sacrificed. Whole brain samples were collected by removing skull and disconnecting optical nerves. Rat brains could be kept in NS basal medium on ice for up to 1 hour. To obtain SVZ cells, brains were cut into coronal slices (see diagram below). SVZ tissue was dissected along the lateral ventricle wall. Collected tissue samples were dissociated into

single cells and plated onto laminin coated dishes in expansion medium described above.


Cortex
cc
Lateral
Ventricle
Striatum
Cell culture
Adult rat brain

3.4.4 Expansion cultures

Rat foetal NS cells could be continuously expanded in conditioned medium. To obtain conditioned medium, cells were plated at relatively high density (50-80% confluence), and culture medium was renewed by 50% every three days. Conditioned medium could be stored at 4 °C for up to one week. Alternatively, rat NS cells could be expanded in fresh expansion medium supplemented with Noggin (20ng/ml, Peprotech). Rat foetal NS cells could be grown on either gelatine or laminin substrates. Neural precursor cells derived from adult rat SVZ were cultured on laminin coated dishes in medium comprising N2 (1x), EGF (10ng/ml), FGF2 (10ng/ml), B27 (1x), and Noggin (20ng/ml). PDGF receptor inhibitor AG1295 (1μM final, Merck) was applied to reduce spontaneous oligodendrocyte generation. Once cultures became confluent, they were split at the ratio of 1:3 with Accutase. Clonal assays of rat foetal NS cells were performed by depositing single cells into each well of 96-well plated, similar to procedures described in 2.4.9.

3.4.5 Generating quiescent neural precursor cells

In the absence of conditioned medium, rat foetal NS cells and adult SVZ neural precursor cells spontaneously became quiescent after ~2 months and 3 weeks expansion respectively. Alternatively, cell quiescence could be induced by applying exogenous BMP4 (10ng/ml, R&D systems) in fresh expansion medium supplemented with EGF and FGF2. Under these conditions, both foetal rat NS cells and adult neural precursor cells would cease division within 7 days.

3.4.6 Differentiation cultures

For neuronal differentiation, 1-2 $x10^5$ rat NS cells were plated into one well of laminin and poly-ornithine coated 12-well plate in expansion conditions. Neuronal differentiation was triggered by removing fist EGF, then FGF2 for 2 weeks. Noggin was optional in neuronal differentiation, as long as the culture medium was renewed by 50% every three

days.

To generate oligodendrocytes, rat NS cells were plated onto Laminin coated dishes in NS basal medium supplemented with FGF2 (10ng.ml), B27 (20ul/ml final), and PDGF-AA (10ng/ml, R&D systems). One week later, medium was switched to NS basal medium supplemented with B27 for another 7 days to allow maturation.

To derive astrocytes, rat NS cells were cultured with 3-5% serum or 10ng/ml BMP4 for 7 days. Culture medium was renewed every 2 days.

3.4.7 Immunostaining

For immunostaining, cells were fixed with 4% PFA for 15 minutes, followed by 30 minutes incubation with BLOCK solution at room temperature. 100ml BLOCK solution contained 97ml PBS, 3ml goat or donkey serum, and 0.1% Triton-X100. After incubation with primary antibodies (2 hours at room temperature or overnight at 4°C, I used Alexa-Fluor secondary conjugates (Invitrogen) and DAPI (Sigma) to visualize the staining. Primary antibodies were used as following dilutions: Nestin (1:20, DSHB), Sox2 (1:400, Chemicon), GFAP (1:300, Millipore), Tuj1 (1:200, Covance), O4 (1:100, R&D Systems), 3CB2 (1:20, DSHB), BLBP (1:500, Abcam), Vimentin (1:20, DSHB), DCX (1:300, Cell Signaling), MAP2 (1:300, Lab version), β-catenin (1:200, BD Biosciences), Chordin (1:200, Santa Cruz Biotech), and phoso-Smad1/5/8 (1:500, Cell Signaling). O4 staining is performed on live cells.

3.4.8 Western Blots

Cell extracts (50 ul) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% NuPage gel, Invitrogen) and electrophoretically transferred to a polyvinylidene difluoride membrane (Amersham). The membrane was incubated with 5%

nonfat dry milk at 4 oC overnight and then probed with the respective antibody (anti-αtubulin, Abcam, 1:2000; anti-Chordin, Santa Cruz Biotech, 1:200, anti-Smad, Santa Cruz Biotech, 1:300). After three washes, the membrane was incubated with 1:5000 dilution of the appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature. Washed blots were subjected to enhanced chemiluminescence.

3.4.9 RT-PCR

I used RNeasy kit (Qiagen) to extract total RNA and Superscript III (Invitrogen) to prepare cDNA, following the instructions provided by manufacturers. An additional DNase step was performed on the total RNA to remove any traces of genomic DNA. Each PCR reaction contained a final concentration of the following: 200μM dNTP mix, 1μM of each primer, 100ng template DNA, appropriate dilution of PCR reaction buffer and 1 unit of Taq DNA polymerase. The reaction condition was set to 30 cycles for all markers except β-actin (25 cycles). Primer sequences and product sizes are listed below.

Table 3.1 Primers used for RT-PCR

Name	Left Primers	Right Primers	Size (bp)
activin a	GATCATCACCTTTGCCGAGT	GTTCACTCCTCTCCCCCTTC	245
activin b	GAGCGCGTCTCTGAGATCAT	TCCACCTTCTTCTCCACCAC	248
activin R1	ACCACCAACGTCGGAGATAG	CCCCTCCACACTTCTCCATA	161
activin R2a	GCAGAAACCATGGCTAGAGG	TAAGGCCAACCCAAAGTCAG	159
activin R2b	CGACTTTGTGGCTGTGAAGA	TCGTTCCACGTGATGATGTT	225
BLBP	CCAGCTGGGAGAAGAGTTTG	TAACAGCGAACAGCAACGAC	192
BMP2	CCCCTATATGCTCGACCTGT	TGTTCCCGAAAAATCTGGAG	240
BMP3	CTCCCCCAAGTCATTTGATG	ATGCTGAGCGAGGACATCTT	174
BMP4	CAGAGCCAACACTGTGAGGA	TATACGGTGGAAGCCCTGTT	199

BMP8	CCTCCACATCAGCATGTTTG	TGTTTGTCCAAGCAGACCAG	238
BMPR1	AGCTGGTTCCGAGAGACTGA	CAGCATGGACTTGGCATCTA	189
BMPR2	CGGCTGTGTGCATTTAAAGA	CACCAATGTGAGACCAGCAC	175
BNF1	CCACACTTACTGCGGTCTCA	GAACATGGATCCTGGGAATG	168
chordin	TGGCCTGGTCTTCGAGTATC	CAGCCGCTGGTAGGAGATAG	205
c-myc	ACGGCCTTCTCTTCTTCCTC	GGTTGCCTCTTTTCCACAGA	173
COCO	TCTGCCCTAGGTAGCTGGAA	GTGGCCAAAACAGAGATGGT	219
cystatin C	TGGTGAGAGCTCGTAAGCAG	TTGCAGCTGGATTTTGTCAG	208
ectodin	GGAGGCAGGCACTTCAGTAG	CGTCTTGTCGTTGACACACC	249
follistatin	TGTTCCAACATCACCTGGAA	GAGCTGCCTGGACAGAAAAC	167
fzd1	AGTCACACCCCCACAGAAAG	CGTGAATCCAGACAAGAGCA	157
fzd2	GAACTCCTGCGCTACTCACC	TCCTCCTGCGAGAAGAACAT	244
fzd4	AACCTCGGCTACAACGTGAC	TGGCACATAAACCGAACAAA	150
fzd9	TCTCACTGGCTTTGTGTTGG	CCGATTTTCACCATCAGCTT	156
GAPDH	AAGGGCTCATGACCACAGTC	GTGAGCTTCCCATTCAGCTC	169
GDF11	ATCCTGGATCTGCACGACTT	TTGGGCCTTCAGTACCTTTG	201
GDF15	CTCCCTCTCTGAGTCCCAACT	GTAGGCTTCGGGGAGACC	223
GDF6	TGCCAGCTTTTTCCAGTCTT	CGATAAAGCCTCAGCTCTGC	168
GDF8	ACGCTACCACGGAAACAATC	GGAGTCTTGACGGCTCTCAG	163
GDF9	CTGATAGGCGAGGTGAGACC	CCGGTCCAGGTTAAATAGCA	230
GFAP	GGTGGAGAGGGACAATCTCA	CCTTCCTCTCCAGATCCACA	155
glast	GGATGGAAAGATTCCAGCAA	ACCTCCCGGTAGCTCATTTT	193
gremlin	GACAAGGCTCAGCACAATGA	CAGGTATTTGCGCTCTGTCA	159
hes1	ACCGGACAAACCAAAGACAG	CTCGGGTCTGTGCTGAGAG	240
lif	TCAACTGGCTCAACTCAACG	ACCATCCGATACAGCTCGAC	177
meteorin	GAGGACCAACGTGCAGAAAT	AGCTCCCTTCCTGGAACAGT	229
nestin	AGAGAAGCGCTGGAACAGAG	AGGTGTCTGCAACCGAGAGT	234
noggin	GAGGTCCTTTCAGCCCTTTC	CCACTTCTCTCCGTCTGCTC	201
olig2	ACCCGATGATCTTTTTCTGC	GGGCTCAGTCATCTGCTTCT	187

p21	AAGAGGCCCAGTACCTCCTC	GGCGCTTGGAGTGATAGAAA	196
PDGF-A	CAAGACCAGGACGGTCATTT	CCTCACCTGGACCTCTTTCA	223
PDGF-B	GAGTGCAAGACGCGTACAGA	GGTCCTCCAGGGTCACTGT	235
PDGFR-α	ACGTTCAAGACCAGCGAGTT	CAGTTTGATGGACGGGAGTT	225
PDGFR-β	CAGGGAAGTGGACTCCGATA	TCCACTGGAAGTTGACCACA	155
PRDC	GTGGCTGTGCTGGTAAAGGT	TGCACCAGTCACTCTTCAGG	187
PTEN	GGAAAGGACGGACTGGTGTA	TGCCACTGGTCTGTAATCCA	197
SDF1	CAGATTGTTGCAAGGCTGAA	CATCTGCAGGAAGCACGTAA	207
shh	CAGGCTTCGACTGGGTCTAC	GAAGGTGAGGAAGTCGCTGT	221
sox2	CACAACTCGGAGATCAGCAA	CTCCGGGAAGCGTGTACTTA	190
TGFβ1	TGAGTGGCTGTCTTTTGACG	GGTTCATGTCATGGATGGTG	185
TGFβ2	GCCCCTGCTGTACCTTCATA	CCAGTCTGTAGGAGGGCAAC	198
TGFβ3	TGTTCACGAGGTGATGGAAA	GCAGTTCTCCTCCAAGTTGC	223
TGFβR1	ACCTTCTGATCCATCCGTTG	AGCTGTCAGCCTAGCTGCTC	160
TGFβR2	GGCTTCACTCTGGAAGATGC	AACAGGTCAGGACTGCTGGT	155
TGFβR3	TTAATTACCCCCAGCTCGAA	TGGTCATTGTCATGGATCGT	232
ventroptin	CAGCAAGTCCTGCGAATACA	GGGAATGCACAGGTCAGTTT	168
wnt1	ACGACTGATCCGACAGAACC	CGGAGGTGATTGCGAAGATA	191
wnt11	ACCTGCTTGACCTGGAGAGA	GGAGACCGTAGCTGAGGTTG	198
wnt2	GTGTGACAATGTGCCAGGTC	AGCTGAGGAGATGGCGTAAA	232
wnt3a	ACTGCACCACTGTCAGCAAC	ACTCCCGAGAGACCATTCCT	246
wnt4	AACGGAACCTTGAGGTGATG	TCACAGCCACACTTCTCCAG	244
wnt5a	GCAGCACAGTGGACAACACT	TGCAACCACAGGTAGACAGC	150

3.4.10 Derivation of reporter rat NS cell line

I transfected passage 20 Ras2 NS cells derived from rat foetal spinal cord with ScaI linearized pCAGGFPIP plasmid DNA (Clontech) using Nucleofector (Amaxa Biosystems, program A-033). One week after transfection, GFP expressing cells were collected by cell

sorting, re-plated, and expanded in conditioned medium under puromycin selection at 2μg/ml for another 7 days. Viable cells with high level of GFP expression were selected by FACS for further expansion as Ras2-GFP NS cell line.

3.4.11 Growth curves and time-lapse videomicroscopy

I prepared growth curves and time-lapse movies using IncuCyte™ imaging system with built-in software (Essen Instruments).

Chapter 4

Intracerebral transplantation, a preliminary investigation of mouse NS cells *in vivo*

4.1 Introduction

Cell cultures allow the selective enrichments to expand or differentiate neural stem cells or precursor cells *in vitro*. and therefore provide a platform to interrogate mechanisms within stem cell biology. However, one concern in stem cell research is that cultured precursor cells may display a narrower or broader potency that is different from *in vivo* circumstances. In fact, several studies have demonstrated that the differentiation potential of cultured precursors *in vitro* does not predict their behaviours *in vivo*. For example, Winkler et al. reported that EGF responsive neurospheres generate both neurons and glia *in vitro*, but they predominantly differentiated into glial cells in E15 rat forebrain (Winkler

et al., 1998). In addition, although bipotential O2A progenitor cells produce both astrocytes and oligodendrocytes under culture conditions, they only generate oligodendrocytes in neonatal rat brain (Espinosa de los Monteros et al., 1993). Furthermore, although neural stem cells have been isolated in culture conditions, endogenous tripotent clones have not been demonstrated *in vivo*. One explanation for these observations is that, if endogenous neural stem cells do exist, the *in vivo* microenvironments may be distinct from culture conditions that strongly influence the behaviour of neural stem cells. Transplantation studies using precursor cells that have been well characterized *in vitro* would contribute to better understanding of the stem cell niche. In addition, transplantation studies would provide essential information for potential cell-replacement therapies.

4.1.1 Transplantation studies using neurosphere cells

Since neurospheres have been widely applied to expand neural precursor cell *in vitro*, they constitute an important cell source for transplantation studies. For example, murine neurospheres were reported to generate both neurons (Vitry et al., 2001; Ogawa et al., 2002) and glial cells (Hammang et al., 1997; Winkler et al., 1998; Lu et al., 2002; Wu et al., 2002) after being transplanted into rodent brain, spinal cord, or retina. In studies using human neurospheres, Fricker et al showed that human cells exhibited Tau expression in hippocampus, striatum, and neocortex of neonatal rat brain and developed neuronal morphologies according to site of integration (Fricker et al., 1999). Brustle et al injected human neurosphere cells into foetal rat brains, and found donor cells generated neurons, astrocytes, and oligodendrocytes that populated all major compartments of the brain (Brustle et al., 1998). Similar observations of incorporation of human neurosphere cells into host brain were also observed in developing primate neocortex (Ourednik et al., 2001).

Although neurospheres generate a wide range of progeny that integrate and populate various regions of host tissue, careful interpretations are required when attempting to determine the differentiation potential of injected neurosphere cells. Since the cell population within neurospheres are heterogeneous, it is possible that a population of cells may have already differentiated or committed into glial/neuronal lineage before transplantation, which makes it hard to determine whether the *in vivo* differentiation is passive or induced. In contrast, monolayer NS cells are maintained as apparently homogeneously undifferentiated cell lines *in vitro*. NS cells would therefore provide a more stable and reliable source to evaluate stem cell differentiation potential *in vivo*.

4.1.2 NS cells generate neurons expressing interneuron markers in vitro

Previous chapters have shown that monolayer NS cells can be derived from mouse, rat, and human neural tissue at different ages. NS cells are tripotent, but their capacity for generating neurons of different subtypes appears to be limited *in vitro*. In culture conditions, mouse NS cells largely generate neurons expressing GAD67, gamma-aminobutyric acid, and Calretinin (Fig 4.1a, b; Conti et al., 2005; and data not shown), and human/rat NS cells generate Calretinin positive neurons (Fig. 4.1c, d). The expression of above markers suggests NS cell derived neurons may represent a type(s) of neocortical interneurons.

Interneurons and projection-neurons are two major neuron classes in the neocortex. Projection-neurons are represented by pyramidal cells and are excitatory (glutamatergic). Interneurons can be subdivided into two types, spiny stellate cells and smooth nonpyramidal neurons. Spiny stellate cells locate in the middle layers of the cortex (especially layer IV) and are thought to be excitatory. Smooth nonpyramidal neurons reside in all cortical layers and are inhibitory (GABAergic). Morphologically, smooth nonpyramidal neurons can be further subdivided into several types including Martinotti,

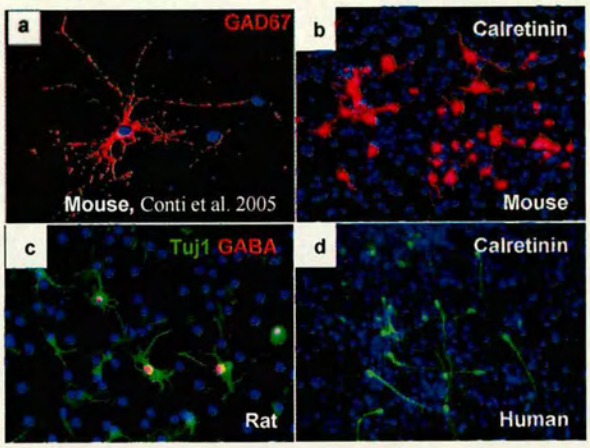


Figure 4.1 NS cells generate neurons expressing inhibitory interneuron markers

In standard neuronal differentiation cultures, mouse, rat, and human NS cells generate neurons expressing inhibitory interneuron markers, including GAD67 (a), Calretinin (b, d), and GABA (c).

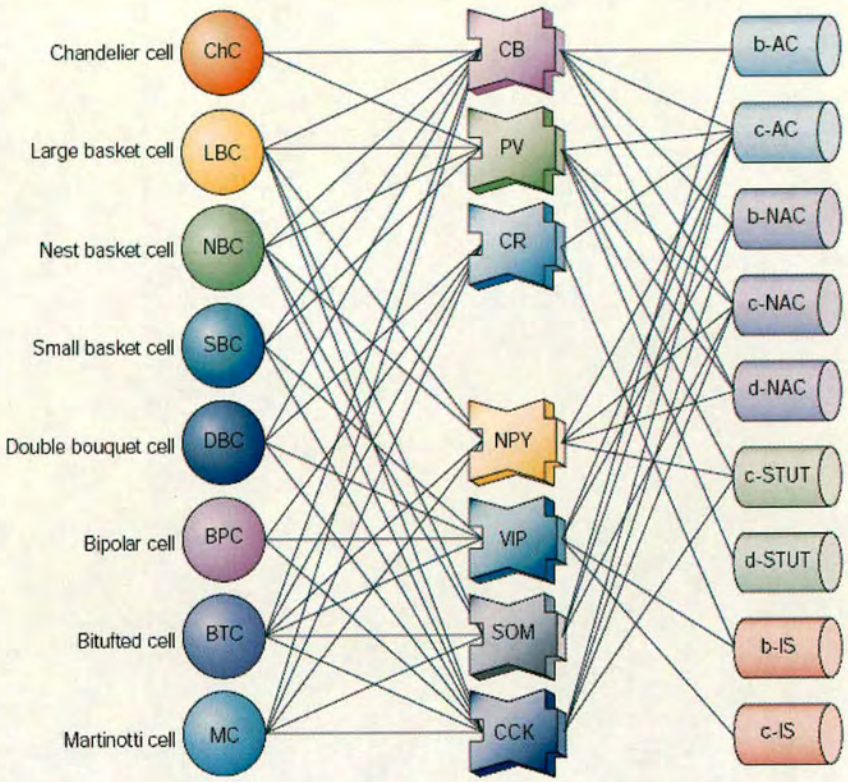


Figure 4.2 Expression of calcium-binding proteins (CBPs) and neuropeptides in interneurons.

Expression profiles of the CBPs calbindin (CB), parvalbumin (PV) and calretinin (CR) and the neuropeptides neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), somatostatin (SOM) and cholecystokinin (CCK) by different morphological and electrophysiological classes of interneuron. AC, accommodating; b, burst subtype; c, classic subtype; d, delay subtype; IS, irregular spiking; NAC, non-accomodating; STUT, stuttering.

Markram et al., 2004

chandelier, double bouquet, large basket, bipolar, neurogliaform, and Cajal-Retzius cells (Markram et al., 2004). It has been shown that calcium-binding proteins (including Parvalbumin, Calbindin, and Calretinin) and neuropeptides (including Somatostatin, neuropeptide Y, vasoactive intestinal peptide, and cholecystokinin) are specifically expressed in morphological subpopulations of neurons belonging to multiple functional systems (Fig. 4.2). Therefore, these calcium-binding proteins and neuropeptides have been widely used as markers to distinguish neuronal subtypes both *in vivo* and *in vitro* (DeFelipe, 1997; Hof et al., 1999; Markram et al., 2004).

To assess whether NS cells may generate multiple neuronal subtypes *in vitro*, I tested a range of modified differentiation conditions (See Chapter 2). However, the only type of neurons that I could detect in mouse/rat/human differentiation cultures was GABA/Calretinin positive neurons. Although it is possible the neuronal differentiation of NS cells is restricted, it is equally possible that *in vivo* microenvironments may provide specific signals that are required to generate different neuronal types. Therefore, transplantation of NS cells into the rodent CNS may better illustrate their differentiation potentials.

4.1.3 Research aims

The research aim in this chapter is to investigate whether mouse NS cells could survive and differentiate *in vivo*. It is necessary to determine the proliferative, migratory, and differentiation capacity of mouse NS cells when they are transplanted into the brain. Since different brain regions and different ages may provide different microenvironments, I injected mouse NS cells into the cortex, striatum, and hippocampus of both adult and neonatal mice. The transplantation studies using mouse NS cells should provide valuable information for future experiments with rat and human NS cells and/or with disease models.

4.2 Results

In order to trace NS cells after transplantation, I used mouse LC1-GFP NS cells that are labelled with retroviral GFP (generated by Dr. Luciano Conti). LC1 NS cells were originally derived from mouse ES cells, and they have been well characterized *in vitro* as a stable and tri-potent mouse NS cell line (Conti et al., 2005). I transplanted naive (undifferentiated) LC1-GFP mouse NS cells into cortex, striatum, and hippocampus of adult and neonatal mouse brains, using ~2 x 10^5 cells for each injection. Animals were sacrificed 6 weeks after transplantation, and brain slices were stained with anti-GFP antibody. In some cases, BrdU solution was injected intraperitoneally 30 minutes before sacrifice to identify proliferative cells.

4.2.1 NS cells survive but do not migrate after transplantation

Mouse NS cells survived well in both adult and neonatal brains 6 weeks after transplantation (Fig. 4.3, 4.4, 4.5). 2-3% of donor cells displayed incorporation with BrdU (Fig. 4.3l), suggesting a proportion of donor cells retained proliferation *in vivo*. In both adult and neonatal brain, the majority of donor cells were restricted in areas close to the injection sites, and the BrdU and GFP double-positive cells usually located along the central line of injection sites. For example, Fig. 4.2a-m illustrated that LC1-GFP cells could only be found within a range of ~1.2mm from the injection sites in both cortex and striatum (Fig. 4.3a-k). Considering the possibility that established cortex and striatum may not favor neural precursor migration, I injected LC1-GFP mouse NS cells into the dorsal and lateral walls of adult ventricle, from which area the endogenous neurogenic progenitors produce young neurons that migrate along RMS into olfactory bulb. However, LC1-GFP cells grafted near SVZ were not migratory either. GFP positive cells could only

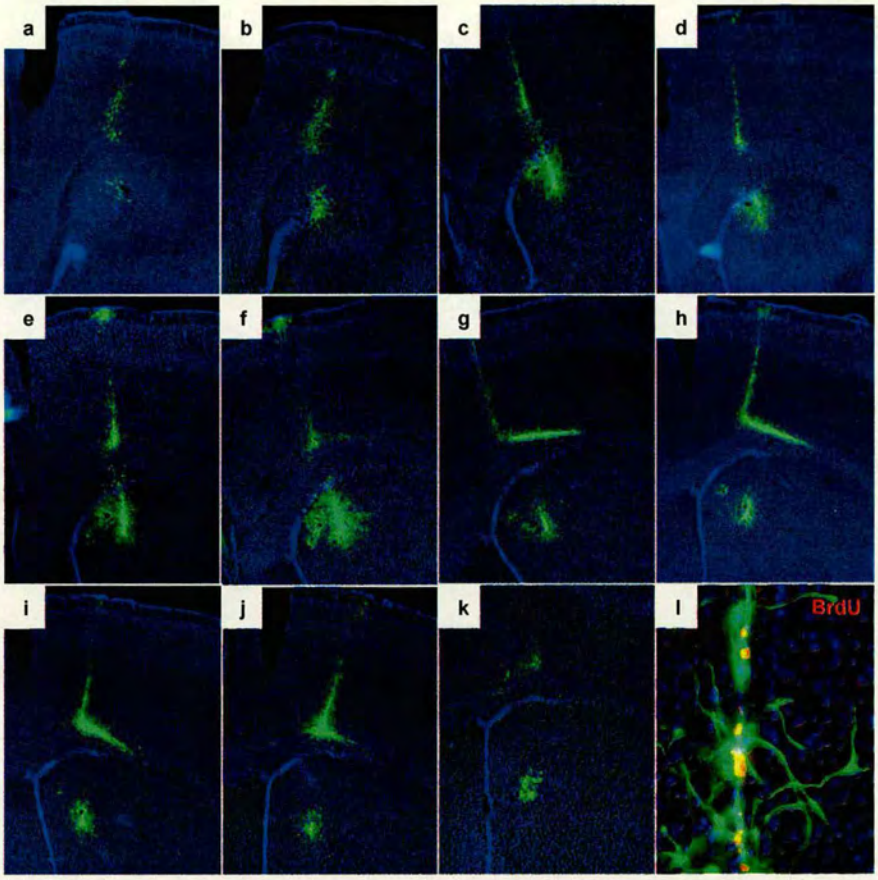


Figure 4.3 Mouse NS cells injected into adult cortex and striatum

Naïve GFP-LC1 mouse NS cells were injected into adult mouse cortex and striatum. Immunostaining on cryo-sections performed 6 weeks after injection indicated that donor cells survived well (a-k). A small number of cells incorporated BrdU injected 30 minutes before sacrifice (l). NS cells displayed limited migration in vivo. GFP labeled cells were detected within 1.2mm from the injection sites (a-k).

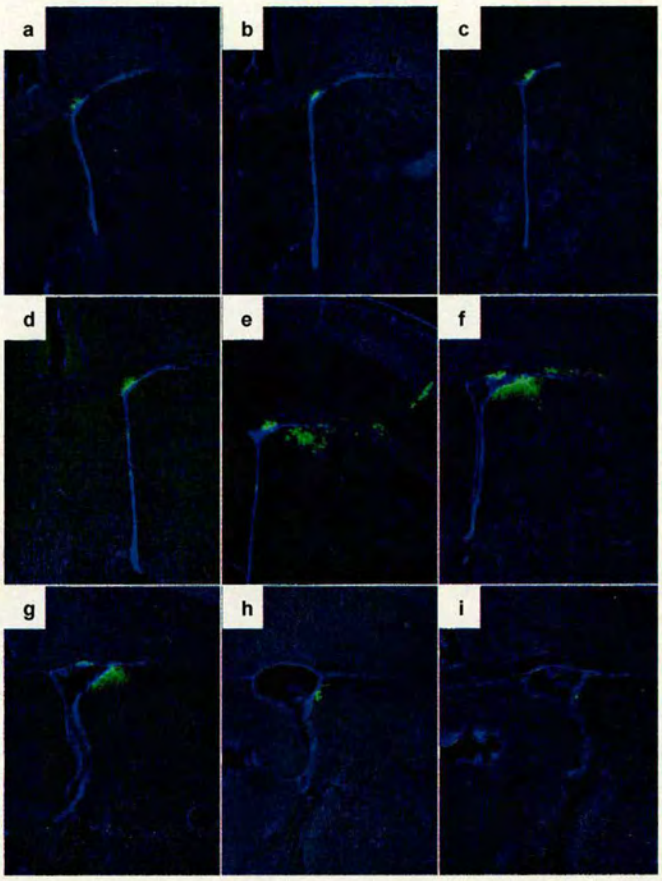


Figure 4.4 Mouse NS cells injected into adult SVZ

Naïve GFP-LC1 mouse NS cells were injected into adult SVZ. Immunostaining on cryo-sections performed 6 weeks after injection indicated that donor cells survived well (a-i). NS cells in SVZ displayed limited migration. GFP labeled cells were detected within 1.35mm from the injection sites (a-k).

be found within ~1.35mm from the injection sites (Fig 4.4 a-i).

4.2.2 NS cells generate morphological neurons and astrocytes in vivo

LC1-GFP NS cells displayed different differentiation potential in adult and neonatal brain. Morphologically, NS cells injected into neonatal cortex, striatum, and hippocampus displayed flat astroglial morphology (Fig. 4.5a-c). Immunostaining indicated that majority of GFP expressing NS cells differentiated into GFAP positive astrocytes (Fig. 4.5d). In contrast, when NS cells were transplanted into adult brain, only a few of them developed GFAP expression, and the majority of cells displayed extended processes similar to neurons (Fig. 4.5 e-h). Immunostaining later indicated that these morphological neurons expressed interneuron markers (see below).

4.2.3 NS cells generate Calretinin and Somatostatin positive neurons in vivo.

Immunostaining indicated that GFP-expressing donor cells with neuronal morphology expressed a different combination of interneuron markers according to site of integration: In adult cortex, the majority of cells expressed Calretinin accompanied with a small number of cells expressing Somatostatin (Fig. 4.6a, d); In striatum, donor cells generated a few Somatostatin positive neuronal cells, but Calretinin expression was not detected (Fig. 4.6b, e); In adult hippocampus, transplanted cells differentiated into Calretinin positive neurons, but no GFP and Somatostatin double-positive cells were detected (Fig. 4.6c, f). LC1 NS cells did not generate Parvalbumin positive neurons at any injection site (Fig. 4.6g-i). Since Calretinin and Somatostatin are expressed by a group of interneurons *in vivo* (Fig. 4.2) (Markram et al., 2004), it was hard to identify which neuron subtype(s) did NS cells generate *in vivo*. More careful investigation with immunostaining and morphological analysis are required in future.

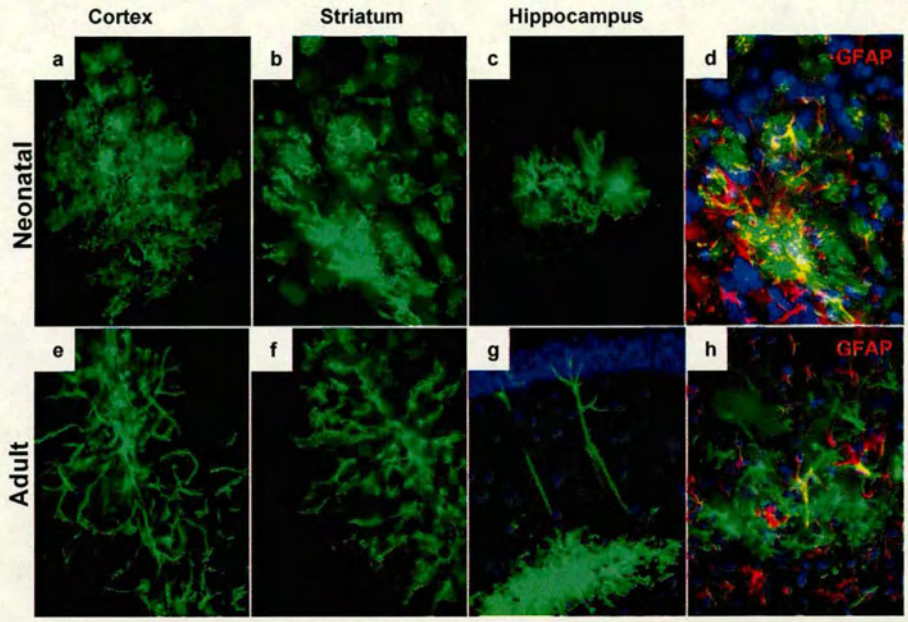


Figure 4.5 NS cells differentiated into morphological neurons and astrocytes *in vivo*

In neonatal cortex, striatum, and hippocampus, majority naïve mouse NS cells differentiated into morphological astrocytes and express GFAP (a-c). In contrast, only a few cells differentiated into GFAP positive astrocytes in when they were injected into corresponding positions of adult brain. Most cells displayed extended processes and appeared to be morphological neurons (e-h).

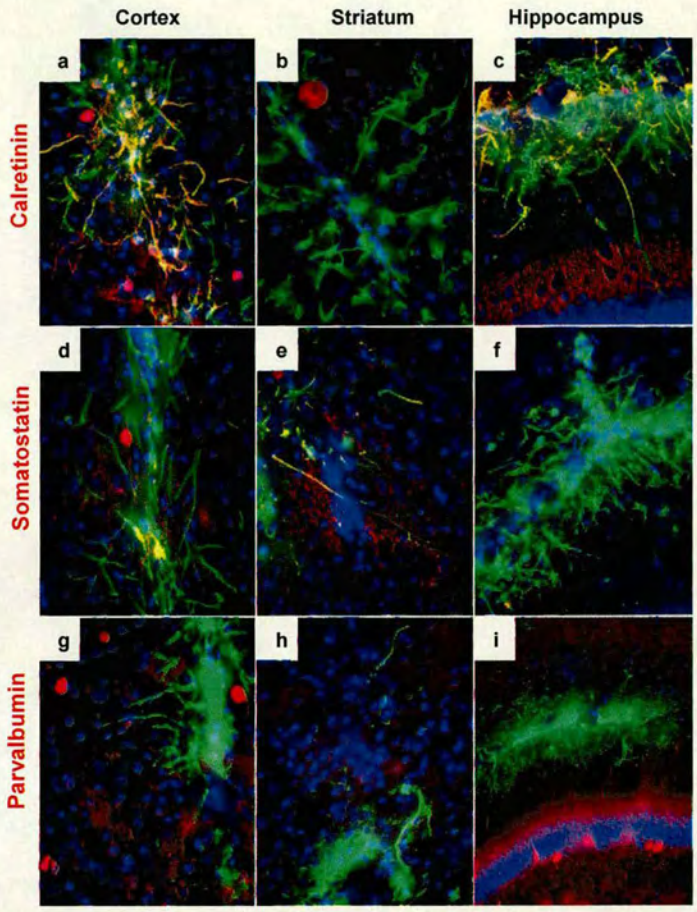


Figure 4.6 NS cells differentiated into neurons expressing interneuron markers *in vivo*

In adult brain, morphological neurons derived from injected mouse NS cells expressed interneuron markers. Calretinin/GFP+ neurons were found in cortex and hippocampus but not in striatum (a-c), while Somatostatin/GFP+ neurons were detected in cortex and striatum but not in hippocampus. Mouse NS cells did not generate Parvalbumin positive neurons (g-i).

4.3 Discussion

In this chapter, I demonstrated that adherent NS cells could be applied in transplantation studies. Mouse NS cells could survive in neonatal and adult brain for at least 6 weeks. They generated both neuronal and glial cells *in vivo*, but their differentiation behaviour was different according to the age of the host and site of injection. This suggests that the microenvironments within brain vary between ages and areas, and that the NS cells have at least some responsiveness to different microenvironments. Since the major peak of endogenous gliogenesis occurs around birth, it is possible that neonatal brain contain a higher level of glia-inducing factors, for example BMPs and/or LIF. In the adult brain, the glial differentiation signals may be reduced, altered, or replaced by other signals. A small proportion of cells retained proliferation 6 weeks after transplantation, indicating some auto- or paracine factor may sustain propagation of NS cells.

Morphological neurons generated from mouse NS cells expressed Somatostatin and/or Calretinin *in vivo*. However, based on the preliminary observations, it is difficult to determine which specific neuronal subtype(s) that mouse NS cells generated *in vivo*. More careful studies are required in future to better characterize the neuronal progeny as well as to exclude the possibility of cell fusion. Moreover, I have not examined whether mouse NS cells are able to generate oligodendrocyte *in vivo*, and whether human and rat NS cells will behave in the same manner as mouse cells.

Although a lot questions remain unanswered, observations in this chapter provide necessary information to design and prepare further transplantation experiments. In fact, ongoing collaborative studies are investigating how human NS cells will behave when they are transplanted into rodent brain. In addition, since to re-populate functional neurons would be a major objective in neural transplantation studies and for potential

cell-replacement therapies, the following strategies might be considered in future to improve the *in vivo* neuronal differentiation efficiency and/or migratory potential: 1) to adjust the microenvironments in host tissue by infusion of growth factors such as EGF, FGF2, or Noggin; 2) to inject pre-differentiated (committed) neuronal progenitors (for example PSA-NCAM positive cells); and 3) To use genetically modified NS cells to generate specific neuron types *in vivo*. Also, disease models may be applied in transplantation studies to examine how NS cell would contribute to the functional restore.

4.4 Materials and methods

4.4.1 Donor cells

I used lentiviral GFP-labelled LC1 mouse NS cells for transplantation (generated by Dr. Luciano Conti, University of Milano). The materials and methods for culturing mouse NS cells can be found in (Conti et al., 2005). Naive LC1 mouse NS cells were harvested by Accutase, and total number of cells was quantified by Hemacytometer. Cells were spin down and re-suspended in a small amount of Hanks BSS to 1-2 x 10^5 cells/μl. Cell samples could then be kept on ice for up to 2 hours before injection.

4.4.2 Animals and anaesthesia

Mouse from two strains, CD1 and 129, were used for transplantations. Animals were bred in Neurosurgery Research, University of California, San Francisco. Neonatal mice at 2 days old and adult mice at 3 months old were selected for injection experiments. Before transplantation, P2 mice were incubated in ice for 3 minutes, and adult mice were injected appropriate volume of anaesthesia (2.2.2-tribromoethanol; Sigma, 20ul per gram) as anaesthesia.

4.4.3 Stereotaxic injection

Animals were fixed on a stereotaxic frame with ear bars and digital readers. A firm incision was made starting from the midline between eyes down to the midline above neck. Bregma (the site the two sutures fuses during development) was located and marked as origin for the stereotaxic coordinates. Injection sites of cortex, striatum, and hippocampus were located by appropriate coordinates (see below), and a hole was drilled in skull for each injection site. ~2 x 10^5 cells in 2ul HBSS were slowly injected through a microsyringe into targeted positions of cortex, striatum, and hippocampus. Skin was sutured after the surgery, and mice were sent back to animal facilities. In

Table 4.1 Transplantation Coordinates (mm, from bregma)

	AP	ML	DV
Cortex	-1.5	1.5	0.6
Striatum	0.5	1.8	2.5
SVZ	0.5	1.0	1.4
Hippocampus	-2.0	1.5	1.75

4.4.4 Perfusion and immunostaining

Six weeks after transplantation, mice were sacrificed by cardioperfusion with 4% PFA. Whole brain tissue was carefully dissected and incubated in 4% PFA at 4°C for 24 hours. Brain slices (20-50um) were prepared using a Leica VT100S vibrating blade microtome. Immunostaining were performed using antibodies at following dilutions: GFAP (Chemicon, 1:500), GFP (1:500, Spring tech), Calretinin (Santa Cruz Biotech, 1:200), Somatostatin (Chemicon, 1:200), Parvalbumin (1:200, Chemicon).

Chapter 5

Conclusion and general discussion

In this thesis, I described the derivation and characterization of human and rat NS cell lines and a preliminary transplantation study using mouse NS cells. My observations indicate that human and rat NS cells can both be maintained in culture conditions as stable cell lines. They are readily induced to produce neurons, astrocyte, and oligodendrocytes in appropriate differentiation conditions and are amenable to genetic modification. Human and rat NS cells are able to generate clonal cell lines, demonstrating their capacity for both self-renewal and multi-lineage differentiation. Unlike neurosphere cultures, adherent cultures allow distinct characterization of both NS cells and their progeny. A good example has been illustrated in Chapter 3 by showing that the responses of rat NS cells to BMP4 and Noggin could be accurately recorded by immunostaining and time-lapse videomicroscopy. In Chapter 4, I demonstrated that NS cells could survive and differentiate into both neurons and glia after transplantation, suggesting these cells could

constitute an invaluable tool for *in vivo* studies.

5.1 Mouse, rat and human NS cells display similarities

Together with the observations from our previous studies (Conti et al., 2005; Pollard et al., 2006), adherent NS cell lines have now been derived from three different species, namely, mouse, rat, and human. Unlike ES cells that exhibit distinct properties between species, mouse, rat, and human NS cells display great similarities. NS cells from three species are similar in terms of the marker expression pattern, the culture requirements, and the self-renewal/differentiation potency (Table 5.1, 5.2, and 5.3). These similarities lead to a speculation that NS cells may represent a generic mammalian stem cell type *in vivo*. More careful studies are required to compare gene expression profile, response to growth factors, and particularly *in vivo* proliferation/differentiation capacity of NS cells between species.

One concern remaining about NS cell lines is that their ability of generating different neuronal subtypes seems limited. Both mouse and human NS cells generally produce GABAergic inhibitory neurons such as Calretinin expressing neurons, regardless whether the NS cells are derived from brain or spinal cord tissue. Although I have tested several differentiating conditions in human NS cell cultures, the attempts for deriving other neuronal subtypes were not successful. I suppose there may be two possible reasons: 1) I have not identified appropriate culture conditions that allow NS cells to differentiate into other cell types; or 2) the differentiation potency of NS cells were partly restricted (committed) that does not allow alternative differentiation. To identify a better differentiating condition, culture supplements such as conditioned medium or tissue extract may be worth to consider. Also, co-culture with astrocytes or other feeder cells could provide necessary factors that lead to a different neuronal differentiation. Alternatively, in the case that NS cells are partly committed in terms of generating multiple neuronal subtypes, deriving new cell lines from different developmental age may

overcome this hurdle. Also, as adherent mouse NS lines have been successfully applied in reprogramming, specific cell types may be derived from more immature cell populations derived from NS cells. In fact, preliminary experiments in our lab have shown that human NS cells can also be reprogrammed to more potent stem cells (unpublished data).

5.2 Quiescent NS cells, a model of quiescent neural stem cells

Although mouse, rat, and human NS cells display similarities, they also exhibit some distinct properties. The most significant difference between rat and mouse/human NS cells is that rat NS cells will spontaneously enter dormancy, generating stellate cells that express astroglial marker GFAP along with neural precursor markers Nestin and Sox2. I have demonstrated that the quiescence of rat NS cells is induced by BMP signals in presence of FGF2 and can be inhibited by BMP antagonist Noggin. Taking together with the observation that BMP alone induces NS cells to generate astrocytes, I speculate that quiescent rat NS cells represent an intermediate cell population between neural stem cells and mature astrocytes. These cells temporarily cease division but retain the capacity to re-enter cell cycle and to differentiate. In other words, they can be considered as quiescent neural stem cells. This speculation is in line with recent evidence that a subpopulation of adult astrocytes act as neural stem cells *in vivo* (Ihrie and Alvarez-Buylla, 2007; Vaccarino et al., 2007). Indeed, preliminary observations from our lab indicated that mouse and human astrocytes derived from NS cells could re-establish proliferative, bipolar, and Nestin positive cell populations after removal of BMP4 or serum and addition of EGF and FGF2 (data not shown). These mouse or human bipolar cells exhibited similar doubling times as mouse or human NS cells respectively. All these stimulate attentions to re-evaluating the function and identity of astrocytes. In future studies, it will be informative to determine the set of markers expression of bipolar cells derived from human or mouse astrocytes as well as their proliferative and differentiation capacity. In addition, whether human and mouse NS cells would also generate quiescent cells in

response to BMP4, FGF2 and EGF need to be explored.

As discussed in Chapter 3, quiescent rat NS cells to some degree display similarities to SVZ Type B cells, which are considered to be adult neural stem cells (see Chapter 1). If this is the case, rat NS cells would therefore offer a readily accessible model to investigate adult neural stem cells *in vitro*. However, before any meaningful conclusion is made, the relationship between quiescent NS cells and endogenous adult neural stem cells requires careful evaluation.

5.3 NS cells in regenerative medicine and drug screening

A range of human nervous system disorders have been identified as neurodegenerative diseases due to the reduction of specific neuronal populations. For example, the degeneration of dopaminergic neurons in substantia nigra, GABAergic neurons in striatum, motor neurons in spinal cord, and cholinergic neurons in cortex lead to Parkinson's, Huntington's, amyotrophic lateral sclerosis, and Alzheimer diseases, respectively. In addition, non-specific neuron death also occurs during aging and as a frequent consequence after stroke or other CNS trauma. In all cases, repopulation of specific neurons or reconstruction of functional circuitry in affected areas could contribute to the recovery from the above neural disorders. For many years, stem/precursor cells have been applied in various transplantation studies to investigate whether or to what extent donor cells may compensate for neuronal degeneration and restore functions. A wide range of cell populations have been tested as donor cells, including primary foetal/adult neural progenitors, ES cells, immortalized precursors, and presumptive transdifferentiation-competent non-neural cells, but significant variations were observed in terms of proliferation and differentiation after transplantation (Conti et al., 2006; Scheffler et al., 2006; Kim, 2007). Previous studies and observations in this thesis have demonstrated that NS cells are tripotent neural stem cell populations that can be expanded

in vitro as cell lines. NS cells, especially NS cells from human tissue, would offer a more stable and well characterized cell source for neural transplantation studies and potential cell-replacement therapies. In addition, since PSA-NCAM expressing human neuronal progenitors can be isolated by FACS (see Chapter 2), it would be possible to harvest either undifferentiated NS cells or committed neuronal progenitors for transplantation experiments.

Aside from the transplantation studies, cultured stem/precursor cells also offer a valuable platform for genetic and chemical screening. For example, Diamandis et al have recently reported the screening of molecules that affect neurotransmission pathways (Diamandis et al., 2007). The authors showed that neurosphere proliferation was changed by neuromodulatory agents, and some compounds displayed potent inhibitory effects on cultures enriched for brain cancer stem cells. Since NS cells have been demonstrated to be uniformly undifferentiated stem cells that are readily adapted for multi-well videomicroscopy and are amenable to genetic modification to provide reporter cell lines, they would should be advantageous substrates for high content genetic and chemical screening.

In conclusion, adherent NS cell lines should constitute an invaluable tool in both basic and applied neurobiological research. Studies of NS cells *in vitro* and *in vivo* which would help us to better understand the principles in brain development, brain cancer, and neurodegenerative disease. When applied in drug screening, disease modelling, and potential regenerative medicine, the potential impact of NS cells on clinical medicine could be enormous.

Table 5.1

Culture requirements of mouse/rat/human NS cells

	Mouse	Rat (foetal)	Human
Coating substrate for derivation/expansion	Gelatine or Laminin	Gelatine or Laminin	Laminin
Derivation conditions	EGF+FGF2	EGF+FGF2	EGF+FGF2
Expansion conditons	EGF+FGF2 or EGF only	EGF+FGF2, EGF only untested	EGF+FGF2 or EGF only
Coating substrate for differentiation	Laminin + Poly-ornithine	Laminin + Poly-ornithine	Laminin + Poly-ornithine
Neuronal differentiation	FGF2 only for 7 days, no growth factor for another 7days	FGF2 only for 7 days, no growth factor for another 7days	FGF2 only for 7 days, no growth factor for another 7days
Oligodendrocyte differentiation	(Glaser et al., 2007)	PDGF+FGF2 for 7 days, no growth factor for another 7days	See 2.4.7
Astrocyte differentiation	BMP4 or serum	BMP4 or serum	BMP4 or serum
Quiescence	Not tested	BMP4+EGF+FGF2	Not tested
Additional requirements	–	Conditioned medium or Noggin	B27 in all cultures

Table 5.2

Cell behaviour of mouse/rat/human NS cells

	Mouse	Rat (foetal)	Human
Long-term stable proliferation	Yes	Yes, requires conditioned medium or Noggin	Yes
Doubling time	~24 hours	~48 hours for brain NS cells ~24 hours for spinal cord NS cells	~72 hours
Clonogenic	Yes	Yes	Yes
Normal karyotype	Yes	Not tested	Yes
Neurosphere formation on uncoated plastic	Yes	Yes	Yes
Tripotent	Yes	Yes	Yes
Amenable to genetic modification	Yes	Yes	Yes

Table 5.3

Marker expression by mouse/rat/human NS cells

	Mouse	Rat (foetal)	Human
3CB2	Yes	Yes	Yes
BLBP	Yes	Yes	Yes
CD133	Not tested	Not tested	Yes
CD15 (SSEA1)	Yes	Not tested	Yes
CD44	Yes	Not tested	Yes
GFAP	No	Not expressed in proliferative NS cells, but expressed in quiescent cells	Yes
Glast	Yes	Yes	Yes
Hoxb9	Not tested	Expressed by spinal cord NS cells	Expressed by spinal cord NS cells
Nestin	Yes	Yes	Yes
Olig2	Yes	Yes	Yes
Otx2	Expressed by cortical NS cells, not tested in ES cell derived NS cells	Expressed by brain NS cells	Expressed by brain NS cells
Pax6	Yes	Not tested	Yes
PDGFRα	No	Yes	No
RC2	Yes	Not tested	Not tested
Sox2	Yes	Yes	Yes
Vimentin	Yes	Yes	Yes

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Appendix I: Human NS cells generate mature neurons defined by electrophysiological attributes

Patch-clamp recordings of neurons derived from human NS cells

In order to examine whether human NS cells generate functional neurons, Dr. Mauro Toselli and Dr. Gerardo Biella in the University of Pavia (Italy) investigated the electrophysiological attributes of derived neurons. Whole-cell voltage–clamp recordings were performed under physiological ionic conditions. Fig. S1b shows current recordings obtained during whole-cell voltage-clamp steps to depolarizing test potentials (protocol shown in Fig. S1a). ~45% of cells were able to elicit a voltage-gated fast Na^+ current and delayed rectifier K+ current, consistent with the observation that 40-50% of the cells express neuronal markers after differentiation. The averaged Na+ current density measured at 0 mV was -30 ± 3.2 pA/pF, whereas the mean value of the membrane capacitance was 37 ± 14.5 pF (n = 5). The inward component of the total ionic current could be blocked by 1 μm TTX, suggesting it was mediated by ions flowing through TTX-sensitive Na^+ channels (data not shown). After switching from voltage- to current-clamp mode, the same cells were able to elicit a supratreshold depolarizing current step followed by regenerative responses, which were identified as overshooting action potentials (Fig. S1c). Therefore, human neurons derived from NS cells are electrophysiologicaly active, display underlying voltage-gated Na+ conductance, and exhibit excitability, all of which are typical properties for mature nerve cells.

Materials and methods

To determine the electrophysiological attributes of neurons from human NS cells, they

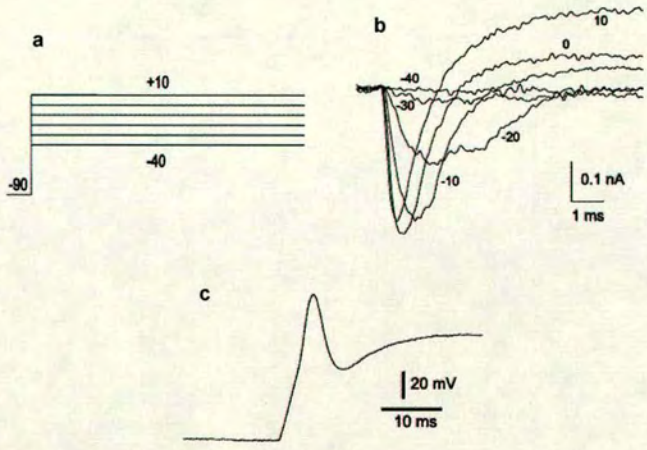

Figure S1 Human NS cells generate mature neurons

Human NS cells generate mature and functional neurons defined by electrophysiological attributes. In voltage–clamp recordings, depolarizing voltage steps between -80 and +10 mV from a holding potential of -90 mV (a). An example of current traces are presented in (b). Voltage response from the same cell are presented in (c). Cells were stimulated with a rectangular current pulse of 80 pA amplitude.

prepare seals between electrodes and cells in a bath solution consists of 155mM NaCl, 1mM CaCl2, 1mM MgCl2, 3mM KCl, and 10mM HEPES/NaOH (pH 7.4). In both voltage-clamp and current-clamp recordings, the pipette filling solution contains 128mM KCl, 10mM NaCl, 11mM EGTA, and 10mM HEPES/KOH (pH 7.4). 1 μM TTX is applied through a multi-barrel perfusion system. We use an Axopatch 200B amplifier (Axon Instruments) to config whole-cell voltage-clamp recordings, and a Digidata 1322A A/D converter (Axon Instruments) to digitize ionic currents. Stimulation, acquisition, and data analysis are carried out using PCLAMP 9 (Axon Instruments) and ORIGIN (Microcal Software) platforms. In voltage-clamp recordings, capacitive and leak currents are reduced by analogue circuitry and removed by P/4 method. They use borosilicate glass to prepare patch pipettes with a final 2-4 MΩ resistance.

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Long-term tripotent differentiation capacity of human neural stem (NS) cells in adherent culture

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Stem cell lines that provide a renewable and scaleable supply of central nervous system cell types would constitute an invaluable resource for basic and applied neurobiology. Here we describe the generation and long-term expansion of multiple human foetal neural stem (NS) cell lines in monolayer culture without genetic immortalization. Adherent human NS cells are propagated in the presence of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2), under which conditions they stably express neural precursor markers and exhibit negligible differentiation into neurons or glia. However, they produce astrocytes, oligodendrocytes, and neurons upon exposure to appropriate differentiation factors. Single cell cloning demonstrates that human NS cells are tripotent. They retain a diploid karyotype and constant neurogenic capacity after over 100 generations. In contrast to human neurospheres, we observe no requirement for the cytokine leukaemia inhibitory factor (LIF) for continued expansion of adherent human NS cells. Human NS cells can be stably transfected to provide reporter lines and readily imaged in live monolayer cultures, creating the potential for high content genetic and chemical screens.

Introduction

Cultured neural stem cells are attracting increasing interest from neuroscientists as a powerful tool for basic and applied neurobiology. *In vitro* expanded human neural stem cells in principle provide an accessible model system to investigate human neurodevelopment and cell biology. They also offer a renewable resource for neurodegenerative disease studies and would be suitable for pharmaceutical and neurotoxicology screening. In addition, scaleable production of *in vitro* human neurons from stem cell lines is the first step towards their use in regenerative medicine.

Until the late 1990s, the only cell line that could consistently generate human neuronal cells *in vitro* was the teratocarcinoma derived NTERA-2. This is a transformed aneuploid cell line that requires complicated manipulations to induce differentiation (Andrews, 1984; Pleasure et al., 1992). These limitations led to the exploration of alternative sources and approaches to produce human neurons *in vitro*. Foetal brain and spinal cord contain proliferating neural progenitor cells, and are potential sources for deriving *in vitro* cell lines. In 1997, Sah et al. established the first immortalized adherent human foetal neural precursor cell line using retrovirally expressed avian *v-myc* (Sah et al., 1997). Subsequent independent reports used similar strategies (Flax et al., 1998; Villa et al., 2000; De Filippis et al., 2007). Several groups have also explored the possibility of expanding human foetal neural precursors in suspension cultures (Svendsen et al., 1998; Carpenter et al., 1999; Riaz et al., 2002), under which conditions neural precursors form floating aggregates termed neurospheres (Reynolds and Weiss, 1992). However, neurosphere cultures are often accompanied by progressive loss of self-renewal and differentiation capacity (Ostenfeld et al., 2000; Reynolds and Rietze, 2005). In addition, since the cell populations in neurosphere are heterogeneous, it is hard to determine the quantity and identity of neurosphere-forming cells (Suslov et al., 2002; Reynolds and Rietze, 2005; Singec et al., 2006). Other researchers have explored derivation of human neural precursor cells using adherent cultures without genetic immortalization (Palmer et al., 2001; Yan et al., 2007). However, characterization of these monolayer human neural precursors is limited to primary cultures. Their long-term stability and tripotent differentiation capacity have not been demonstrated.

We have reported the establishment of clonogenic mouse neural stem (NS) cell lines derived from both ES cells and foetal CNS.

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Mouse NS cells undergo symmetrical division in adherent cultures and retain multi-lineage differentiation capacity after prolonged expansion (Conti et al., 2005; Pollard et al., 2006a,b). Here we apply this approach to human foetal CNS tissue and report the derivation and characterization of human NS cell lines. Human NS cell lines are clonogenic and highly expandable. They display constant self-renewing and tripotent differentiation capacity after over 100 generations, and retain a diploid karyotype. These findings demonstrate that human foetal NS cell lines are self-renewing human neural stem cells *in vitro* without requirement for a specialised cellular niche or genetic immortalization. As such they may provide a new platform for a range of studies in basic and applied human neurobiology.

Results

Derivation and expansion of adherent human NS cells

The source that we have employed to derive human NS cells is human foetal neural tissue at embryonic 50–55 days, equivalent to Carnegie stages 19–22. Human foetal cortex was carefully dissected and dissociated into single cells by incubation with Accutase. Primary cells were then seeded onto laminin coated dishes in growth medium containing both EGF and FGF2. Cells readily attached and produced a morphologically heterogeneous population containing both neural precursors (Nestin+) and neurons (Tuj1+) (Fig. 1Aa–c). To enrich for undifferentiated neural precursor cells, on day 7 after plating, we temporarily transferred primary human cells onto gelatin coated dishes, as under these conditions, neurons and committed neuronal progenitors fail to survive. Seven to 10 days later, viable precursor cells were re-plated back onto laminin substrate for further expansion. The cell population was then taken over by proliferating neural precursor cells. Three weeks after (initial) plating, immunostaining indicated that the primary human culture was homogeneously Nestin positive and Tuj1 negative (Fig. 1Ad–f). At this stage, cultures were considered to passage one human NS cell.

Once established, human NS cells can be expanded continuously in monolayer culture. On laminin substrate, the doubling time of human NS cells is 2–3 days, slower than mouse NS cells grown in the same condition (Conti et al., 2005; Pollard et al., 2006a,b). We routinely split human NS cells 1:2 to 1:3 every 5–7 days. Proliferating human NS cell cultures contain both small bipolar cells and more flattened apolar cells. Time-lapse videomicroscopy reveals dynamic interconversions between these morphologies. The cells are also highly motile (Fig. 1Ba and Video 1). Some elongated

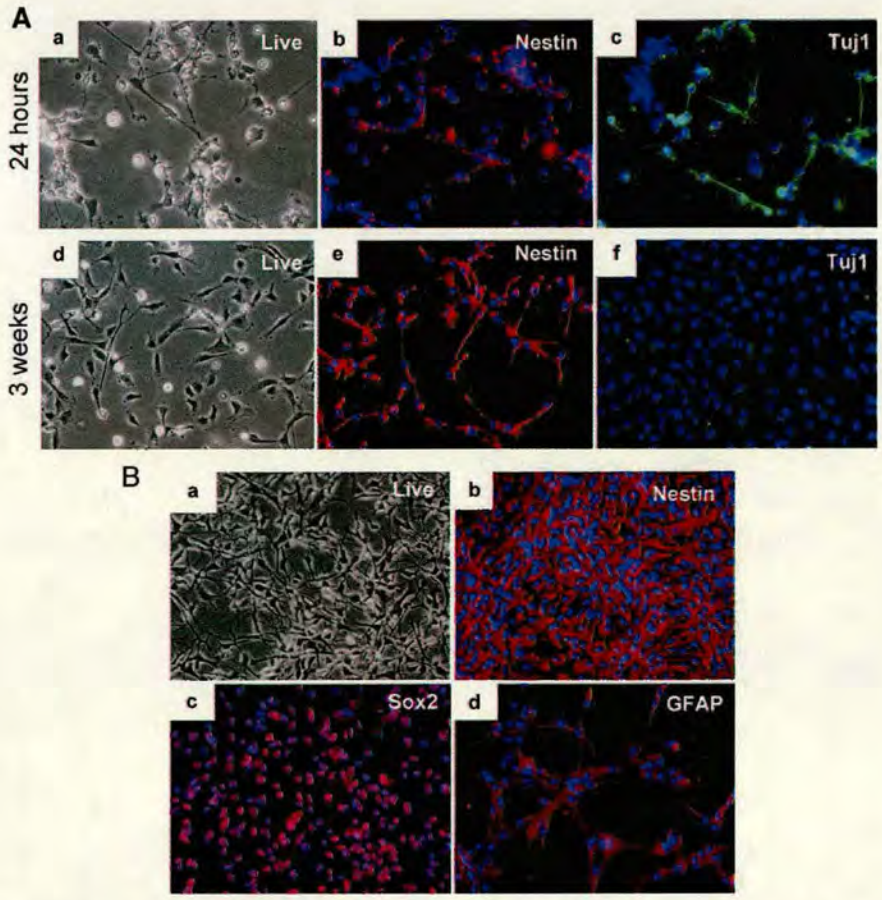

Fig. 1. Derivation and expansion of human NS cells. (A) *Primary cultures*. Primary cells from human foetal forebrain were plated onto laminin coated plastic. Immunostaining performed 24 h later indicated a heterogeneous cell population containing neural precursors (Nestin+) and neurons (Tuj1+) (a–c). After 3 weeks of expansion, almost all cells were Nestin positive and Tuj1 negative (d–f). (B) *Expansion cultures*. Human NS cells can be expanded continuously in the presence of both EGF and FGF2 (a). NS cells uniformly express Nestin and Sox2 (b, c), and show weak staining with GFAP antibody (d).

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cells show interkinetic nuclear migration as previously noted for mouse NS cells (Conti et al., 2005).

To investigate whether these human NS cell culture conditions can be used reproducibly, we repeated the derivation and expansion experiments using 8 independent tissue specimens from human foetuses at embryonic days 50–55. Human NS cell lines could be generated from all specimens in which the neural tissue was well preserved. The continuous adherent procedure is more efficient than allowing primary cells to form neurospheres and subsequently isolating NS cells, as described previously (Conti et al., 2005). It takes, on average, one month to derive an adherent and morphologically homogeneous human NS cell population with a total number of ~2 million cells. To date, we have successfully derived 5 human brain NS cell lines, named CB192, CB516, CB525, CB541, and CB660. In addition, human NS cell lines can also be derived from foetal spinal cord using the same culture conditions. Spinal cord NS cells and brain NS cells are morphologically indistinguishable (data not shown). We have derived 4 human foetal spinal cord NS cell lines, named CB516SP, CB525SP, CB540SP, and CB660SP. To date, CB192, CB516, CB541, CB660, CB516SP, and CB660SP NS cell lines have been expanded for over one year to passage ~50. None displayed cell senescence, crisis, or spontaneous differentiation.

In expansion culture, human NS cells homogeneously express immature neural precursor markers Nestin and Sox2 (Fig. 1Bb, c). In contrast to mouse NS cells, human NS cells are weakly immunoreactive to anti-GFAP antibodies (Fig. 1Bd), consistent with the known activity of the human GFAP promoter in radial glia progenitor cells (Malatesta et al., 2000; Rakic, 2003). Real-time PCR shows that the level of GFAP mRNA expression in human NS cells is much lower than human astrocytes derived from NS cells upon serum or BMP exposure. Furthermore, human NS cells do not express the astrocyte and ependymal cell marker S100β (Fig. 2). Expression of neuronal markers (DCX, Tuj1, or MAP2) is not detected in expansion conditions (data not shown).

Human NS cells generate neurons, astrocytes, and oligodendrocytes

Neural stem cells, as a type of tissue stem cell, are anticipated to be able to generate the three major cell types of the CNS: neurons, oligodendrocytes, and astrocytes. The differentiation potential of human NS cells was assessed using protocols previously developed for mouse NS cells (Conti et al., 2005; Glaser et al., 2007).

We plated ~1×10^5 cells into poly-ornithine and laminin coated 35-mm dishes for differentiation cultures. Neuronal differentiation was triggered by removing EGF from growth medium. Over 14 days, cells began to form small aggregates (still adherent) and developed elongated spindle processes, accompanied by reduced proliferation and some cell death (Video 2). Although some cells at this stage presented neuronal morphology and doublecortin (DCX) expression (see below), the absence of Tuj1, MAP2, or neurofilament expression suggests these cells were immature neuronal progenitors. For further differentiation and maturation, FGF2 was withdrawn and cultures were maintained for another 2–3 weeks. By the end of 4th week of neuronal differentiation, many cells became Tuj1 positive and exhibited thin elongated processes. On the basis of 8562 cells examined in four separate experiments, 43+ 4.6% of cells were Tuj1 positive (Fig. 2a and Video 2). The majority (76±3.7%) of Tuj1 positive neurons also express neurofilament, a marker for mature neurons (Fig. 2b). Most non-neuronal cells (DCX/Tuj1/MAP2/Neurofilament negative) also presented extended morphology, although they retained Nestin/GFAP expression (Fig. 2c). On the basis of 1.2×10^6 cells scored in three separate analyses, flow cytometry indicates that 28±2.3% of intracellularly stained cells exhibited a high level of GFAP expression at the end of the differentiation culture, suggesting that a proportion of human NS cells differentiated into astrocytes (Fig. 2c, d). No O4 (oligodendroglial marker) expression could be detected in these conditions (data not shown). We find that all human NS cell lines retain stable and robust neurogenic capacity after extended expansion. For example, CB192 NS cells at passage 42 (after nearly one year expansion) were still able to generate 39± 5.1% Tuj1 positive neurons (Fig. 2e).

To generate oligodendrocytes, cells seeded on poly-ornithine/laminin coated dishes were treated with DMEM/F12 medium supplemented with N2, forskolin, FGF2, and PDGF for 2 weeks (Glaser et al., 2007). From the third week, medium was changed to DMEM/F12 medium supplemented with N2, PDGF, T3, and ascorbic acid (Glaser et al., 2007). Another 7 days later, PDGF was withdrawn from the culture. At the end of the 5th week, 1–2% O4 positive cells with branched oligodendrocyte morphology could be detected in culture (Fig. 2f) along with 16±3.9% Tuj1 positive neurons (data not shown). The majority of cells remained O4 negative and Nestin positive, but up to 30±2.3% expressed NG2 (data not shown), a marker associating with oligodendroglia progenitors.

In the absence of EGF and FGF2, a morphologically homogeneous astrocyte population can be derived from human NS cells after 2 weeks culture with BMP4 or serum. Time-lapse video shows that, upon exposure to serum, the cell migration and proliferation attenuate significantly (Video 3). Unlike NS cells, astrocytes present flat sheet-like morphology with large nuclei. They are negative for Nestin and Sox2, but they express the astroglial marker S100β and a high level of GFAP (Fig. 2g–i). Simply removing EGF and FGF2 from expansion medium without addition of BMP or serum also leads to NS cell differentiation into astrocytes. However, significant cell death occurs under such conditions. Recently it was reported that different types of human astrocytes may express distinct GFAP isoforms. For example, adult subventricular zone (SVZ) astroglial cells express GFAPδ, while most other astrocytes express GFAPα (Roelofs et al., 2005). In our cultures, both human foetal NS cells and derivative astrocytes express GFAPα (Fig. 2h), but the level is considerably higher in the differentiated astrocytes.

Culture requirements for human NS cell self-renewal

The above observations suggest that human NS cells are expandable and tripotent stem cells. We then tested a series of conditions to define the culture requirements for human NS cell self-renewal.

EGF and FGF2 are mitogens widely used in neural stem cell cultures. During derivation (the first 4 weeks after cells are plated), primary human cells attached on laminin substrate within 24 h and started to proliferate in the presence of both EGF and FGF2 (Fig. 3Aa). However, in medium containing only one of the growth factors, no extended cell proliferation was observed, and NS cell lines could not be established (Fig. 3Ab, c). In addition to EGF and FGF2, we find that laminin substrate and B27 supplement are important for efficient human NS cell derivation. Primary cells grown on gelatin coated or uncoated dishes easily detach and tend to form neurospheres (Fig. 3Ad), resulting in slower proliferation as described in our previous study (Conti et al., 2005).

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During long-term expansion (>1 year), the continuous proliferation of human NS cells is also optimal on laminin substrate with both EGF and FGF2 (Fig. 3Ba–c). Switching laminin substrate to gelatin or uncoated plastic leads to cell detachment (Fig. 3Bd–f). Transfer to FGF2 only leads to neuronal differentiation accompanied by attenuated proliferation and some cell death (Video 2). In medium containing EGF alone, neuronal and glial differentiation remain fully suppressed, but cells exhibit a slower proliferation rate with a doubling time of 3–4 days (Fig. 3Bg–i). When human NS cells expanded in EGF only are exposed to differentiation conditions described above, they are able to generate both neurons and glial cells. These observations mirror our previous findings with mouse NS cells, where addition of FGF2 is essential for initial derivation but can be dispensed with during subsequent propagation (Pollard et al., 2006b).

Studies on human neurospheres have suggested that LIF is important for long-term stability of human progenitors (Carpenter et al., 1999; Wright et al., 2003). In order to investigate the effects

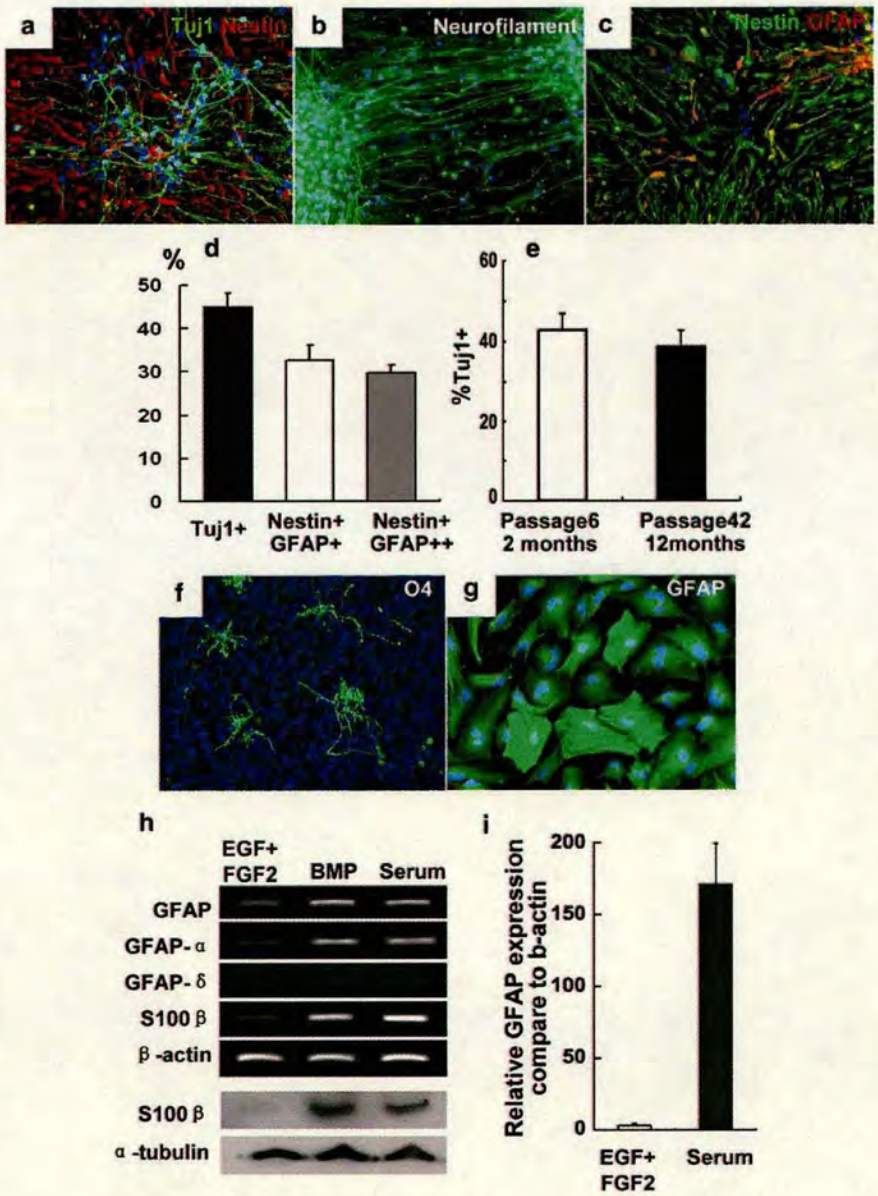

Fig. 2. Human NS cells are tripotent. Over 40% of cells express neuronal marker βIII-tubulin (Tuj1) and Neurofilament one month after mitogen withdrawal (a, b). Remaining cells can be stained with Nestin and/or GFAP antibodies (c). Flow cytometry indicates the percentage of immunopositive cells after 30 days differentiation (d). Human NS cells retain robust neurogenic capability after nearly one year expansion, generating 39±5.1% Tuj1+ cells, similar to cells at early passages (43±4.6%) (e). When treated with PDGF, FGF2, forskolin, and T3, human NS cells are able to generate O4+ oligodendrocytes (f). Human NS cells generate a pure astrocyte population with flat sheet-like morphology upon exposure to serum or BMP4 (g). RT-PCR and real-time PCR show that astrocytes express a much higher level of GFAP (GFAPα) than NS cells (h, i). RT-PCR and western blot indicate that human NS cells express little or no S100β compared with astrocytes (h).

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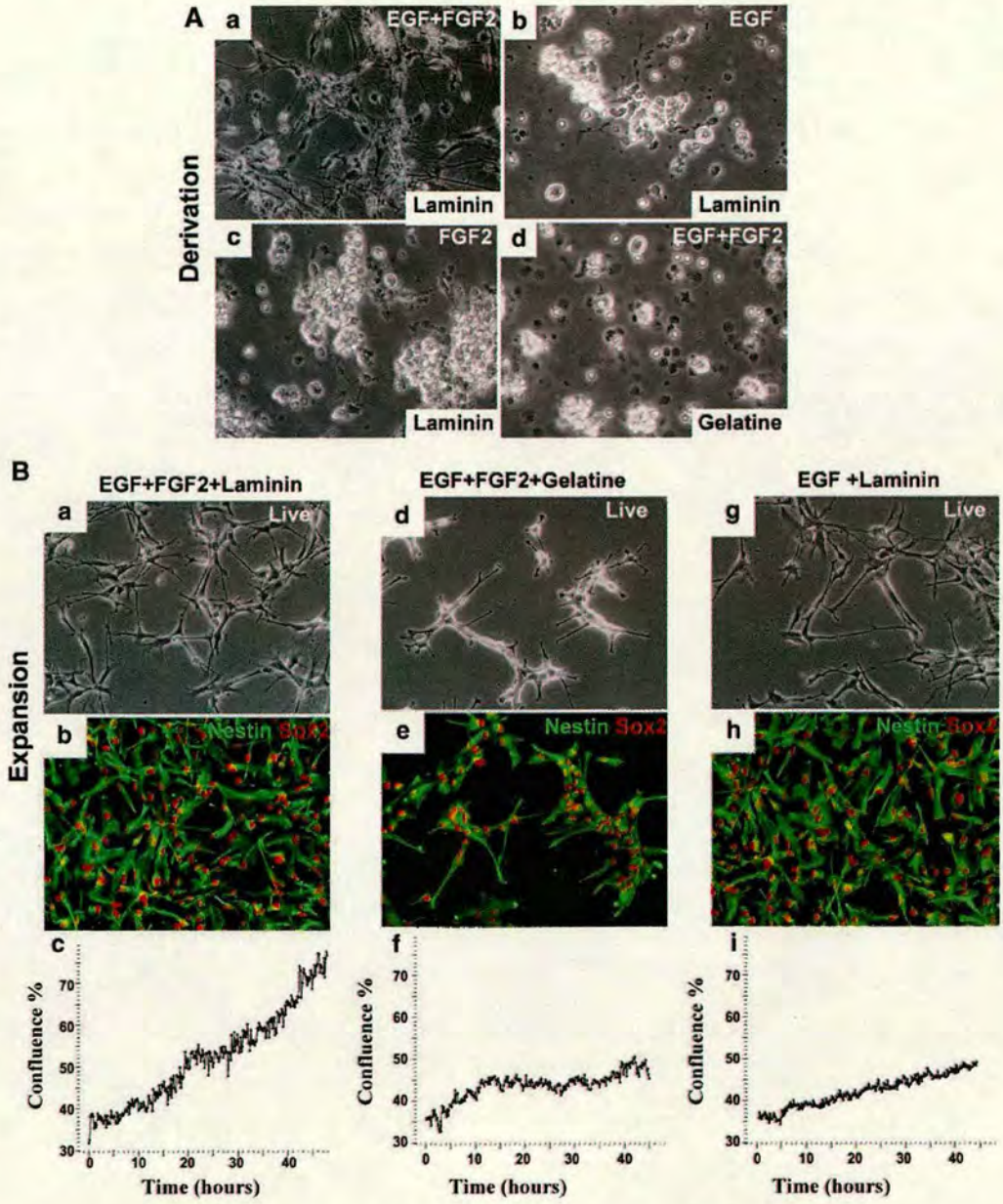

Fig. 3. Characterization of culture conditions for human NS cells. (A) *Derivation*. Efficient derivation of human NS cells requires a combination of EGF, FGF2, and laminin coating (a). No significant proliferation can be obtained in primary cultures using EGF or FGF2 alone (b, c). Primary cells tend to detach and form neurospheres on gelatin coated dishes (d). (B) *Expansion*. In the presence of EGF and FGF2, Nestin+/Sox2+ human NS cells have a doubling time of 2–3 days on laminin coated dishes (a–c). Replacing laminin with gelatin substrate results in significant cell detachment and reduced rate of expansion (d–f). Removing FGF2 from expansion medium leads to slower expansion, but cells retain Nestin/Sox2 expression (g–i).

of LIF on monolayer human NS cells, we performed parallel experiments throughout derivation, expansion, and differentiation procedures. At all stages, cells cultured with recombinant human LIF were not distinguishable to those without. Furthermore, RT-PCR indicates that human NS cells do not express GP130 (data not shown), and all our extended passaging studies were carried out without LIF. This indicates that LIF makes no contribution to long-term NS cell propagation. The reported effects of LIF on human neural progenitors may not be direct and likely relate to the heterogeneity of the neurosphere culture system.

Long-term stability of clonogenic human NS cell lines

Using the culture conditions described above, human foetal NS cells can be maintained as stable cell lines. However, progenitor cells with limited differentiation capacity could also exhibit proliferative phenotypes, and the multi-lineage derivatives in bulk culture may come from distinct unipotent progenitor cells. In order to determine the potency of human NS cells, we performed clonal analyses. Single human NS cells were deposited into a 96-well plate using a flow cytometry cell sorter. After 4-week expansion, 8 of 96

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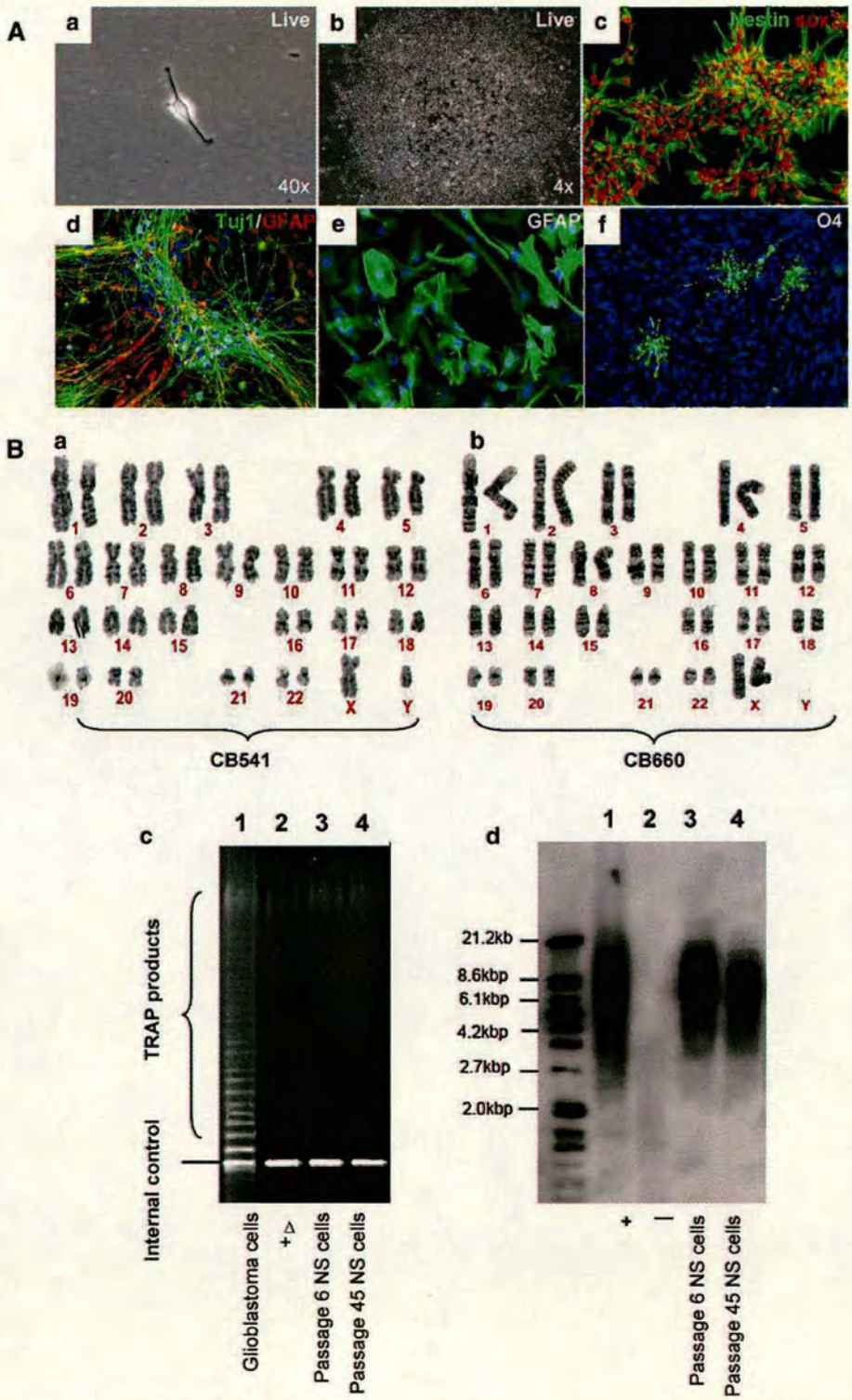

Fig. 4. Clonogenicity and cytogenetic stability of human NS cells. (A) *Clonal human NS cell lines*. Clonal cell lines derived from single human NS cells retain uniform Nestin and sox2 expression (a–c). Clonal NS cell lines are tripotent, generating neurons, astrocytes, and oligodendrocytes in appropriate differentiation conditions (d–f). (B) *Cytogenetic stability*. CB541 (passage 45) and CB660 (passage 42) human NS cells retain normal diploid karyotype (CB541: 46, XY; CB660: 46, XX) (a, b). A TRAP assay shows that telomerase-mediated 6-nucleotide ladder products cannot be detected in human NS cells at either early (sample 3) or late passages (sample 4), but such products are evident in human glioblastoma cells (sample 1) (c). The telomeric length of human NS cells at early (sample 3) and late passage (sample 4) is measured using a non-radioactive chemiluminescent assay. Control DNA (provided by the manufacturer) and reaction buffer (no DNA) were prepared as positive (sample 1) and negative (sample 2) controls. The mean length of terminal restriction fragments (TRF) are: sample 1: 10.2 kbp; sample 3: 9.8 kbp; sample 4: 7.2 kbp (d).

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deposited single cells generated colonies (Fig. 4Aa, b). We expanded two colonies to establish clonal NS lines named CB192-1 and CB192-2. Both cell lines display extensive similarities to their parent cell line CB192. They show stable proliferation and uniform Nestin/sox2 expression in expansion medium (Fig. 4Ac). Both lines are able to generate neurons, astrocytes, and oligodendrocytes in the respective differentiation conditions (Fig. 4Ad–f).

One concern in cell culture is that proliferative cell populations may undergo genetic transformations, resulting in circumvention of cell cycle regulatory mechanisms and manifesting in an immortalized phenotype. Genetically immortalized cells are frequently marked by irregular/hyperplasic growth rate, abnormal chromosome numbers, and/or robust telomerase activity. In human NS cell cultures, cells display a constant doubling time of 2–3 days during

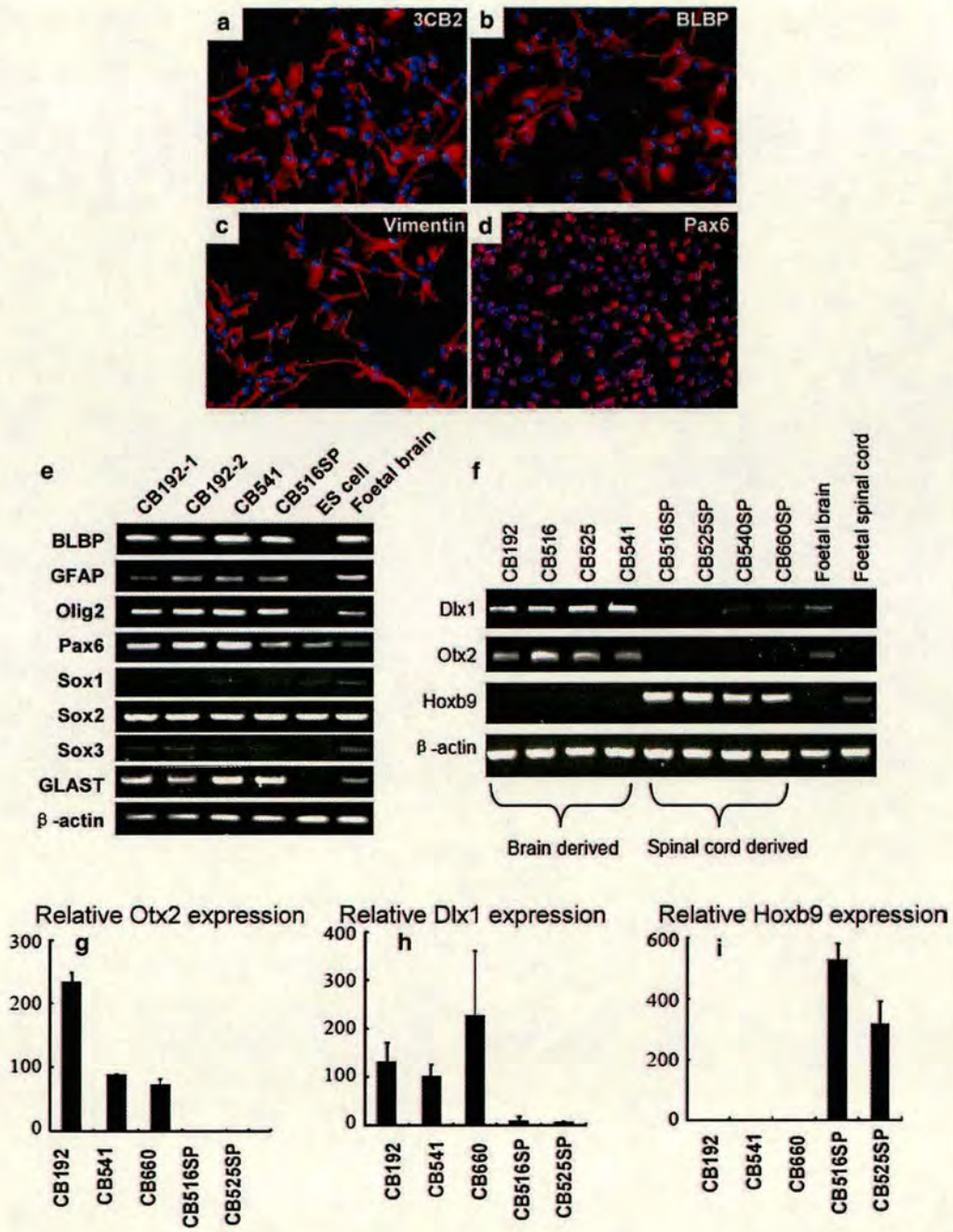

Fig. 5. Human NS cells express radial glia markers and retain a subset of regional markers. Immunostaining shows that human NS cells express 3CB2, BLBP, Vimentin, and Pax6 (a–d). RT-PCR indicates that independent human NS cell lines express the same pattern of neural precursor/radial glia markers. CB192-1 and CB192-2 are clonal lines. CB541 and CB516SP NS cells are derived from foetal brain and spinal cord respectively (e). RT-PCR and real-time PCR show that human brain NS cells (CB192, CB541, CB525, CB660) express Otx2 and Dlx1 mRNA, while spinal cord cells (CB516SP, CB525SP, CB540SP, CB660SP) express Hoxb9 (f–i).

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long-term expansion. Their proliferation remains fully dependent on exposure to EGF and FGF2. We analyzed metaphase spreads from three independent samples (CB192, CB660, and CB541) after at least 40 passages (over 100 generations). Most spreads (18 of 20, 9 of 10, and 9 of 9 respectively) displayed a euploid number of chromosomes. Karyotypes of two samples, CB660 and CB541, were illustrated in Fig. 4B. Human NS cells do not display telomerase activity (Fig. 4Bc), and the average length of telomeres in human NS cells shortens during expansion, from ~9.8 kbp at passage 6 to ~7.2 kbp at passage 45 (Fig. 4Bd). These findings demonstrate that human NS cells are different from tumor cells (Fig. 4Bc). However, it also implies that, although human NS cells are highly expandable, they may undergo replicative ageing during very extensive expansion.

Human NS cells exhibit similarities to radial glia and retain a subset of regional markers

An important issue to consider when characterizing stem cells *in vitro* is whether these cells have *in vivo* counterparts. Our previous study indicates that mouse NS cells exhibit similarities to radial glia (Conti et al., 2005; Pollard et al., 2006a,b), which generate both neurons and glia in the developing brain (Malatesta et al., 2000; Noctor et al., 2001; Merkle et al., 2004). Immunostaining and RT-PCR reveal that human foetal NS cells express a set of markers including brain lipid binding protein (BLBP), 3CB2, astrocyte-specific glutamate transporter (GLAST), Vimentin, and GFAP (Fig. 5a–c, e), which are hallmarks for radial glia cells. In addition, human NS cells express neural progenitor markers including Nestin, Sox2, Pax6, Olig2, and CD133 (Prominin) (Figs. 5d, e and 6). Sox1 is transiently expressed in mouse and human neural precursor cells, but is not maintained in NS cells (Conti et al., 2005; Lowell et al., 2006). Similarly, human foetal NS cells do not express Sox1.

In terms of transcription factors representing regional identities, NS cell lines derived from whole foetal forebrain express Otx2 and Dlx1. In contrast, cell lines derived from foetal spinal cord do not express Otx2 or Dlx1, but they express Hoxb9 (Fig. 5f–i), suggesting some level of regional identity may be retained in NS cells from different sources. Expression of other regional markers Emx1/2, Gsh1/2, and Dlx2 is not detected in either brain or spinal cord human NS cells.

Cell population transitions during neuronal differentiation

Early stages of neuronal differentiation from human neurospheres are difficult to visualise and study due to multicellularity and heterogeneity. Adherent human NS cells allow direct inspection of both stem cells and their differentiating progeny. We employed videomicroscopy, time-course immunostaining, and RT-PCR to track the cell population transitions during neuronal differentiation and begin to define the properties of intermediate neuronal progenitor cells.

As described above, the protocol for *in vitro* neuronal differentiation of human NS cells consists of 2 weeks of FGF2 induction followed by 2–3 weeks of maturation in basal medium without growth factor (Fig. 6a–c and Video 2). During weeks 1–2, immunostaining shows expression of neural stem cell surface marker CD133 decreases sharply, while the expression of polysialylated neural adhesion cell molecule (PSA-NCAM) and DCX emerges (Fig. 6d, e, g, h). During weeks 3–4, Tuj1+ cells appear soon after FGF2 withdrawal, and Tuj1 staining overlaps with PSA-NCAM or DCX staining (Fig. 6f, i). Nestin+ cells persist throughout the differentiation procedure, and they constitute the majority of non-neuronal cells (Tuj/DCX/PSA-NCAM negative) at week 4 (Fig. 6j–l). RT-PCR confirms the decrease of CD133 and increase of DCX during neuronal differentiation (Fig. 6m). In addition, RT-PCR indicates that Olig2 expression also decreases soon after the onset of differentiation (Fig. 6m). Fig. 6n summarizes the marker expression changes during the 4-week neuronal differentiation.

Isolation of PSA-NCAM positive cells enrich for neuronal progenitors in differentiating NS cultures

Although human NS cells retain neurogenic capacity over long-term *in vitro* expansion, only ~40% of cells differentiate into neurons using the current protocol. PSA-NCAM is a cell surface marker that appears to be expressed only by cells undergoing neuronal differentiation (see above). This suggests that PSA-NCAM could be a candidate marker for isolating neuronal progenitors from neuronal differentiation cultures. We therefore assessed whether neuronal progenitors or neurons may be purified from differentiation cultures using PSA-NCAM antibody staining and subsequent Fluorescence Activated Cell Sorting (FACS).

Although neuronal differentiation cultures at later time point (weeks 3–4) contain higher percentage of PSA-NCAM positive cells, the viability of cells after FACS is low at these stages. We therefore performed flow cytometric sorting with CB660 and CB660SP NS cells on day 14 of cultures in FGF2 only (Fig. 7a). Flow cytometry indicated that, among 6×10^5 cells scored in three separate analyses, 6.8±1.1% of cells were PSA-NCAM+ at this time point. Most of these (82.4+5.2% of the PSA-NCAM positive cells) retained CD133 expression. We sorted these cell populations and re-plated them on laminin coated dishes for further differentiation (cultures without mitogen) (Fig. 7a). Two weeks later, only the PSA-NCAM+ cell population generated Tuj1+ neurons. Although the cultures also contained GFAP and Nestin expressing cells at the end of differentiation, the percentage of Tuj1+ neurons generated from PSA-NCAM+ cells could reach 66±5.8% (Fig. 7b). In contrast, most PSA-NCAM negative cells differentiated into astrocytes with strong GFAP staining (Fig. 7c, d). This indicates that PSA-NCAM can be used as a maker to enrich for viable neuronal progenitors.

Human NS cells generate mature neurons

In order to examine whether human NS cells generate mature neurons, we investigated the electrophysiological attributes of derived cells. Whole-cell voltage-clamp recordings were performed under physiological ionic conditions. Fig. 8b shows current recordings obtained during whole-cell voltage-clamp steps to depolarizing test potentials (protocol shown in Fig. 8a). ~45% of cells were able to elicit a voltage-gated fast Na^+ current and delayed rectifier K^+ current, consistent with the observation that 40–50% of the cells express neuronal markers after differentiation. The averaged Na^+ current density measured at 0 mV was −30± 3.2 pA/pF, whereas the mean value of the membrane capacitance was 37±14.5 pF (n=5). After switching from voltage- to current-clamp mode, the same cells were able to elicit a supratreshold depolarizing current step followed by regenerative responses, which were identified as overshooting action potentials (Fig. 8c). Therefore, human neurons derived from NS cells are electro-

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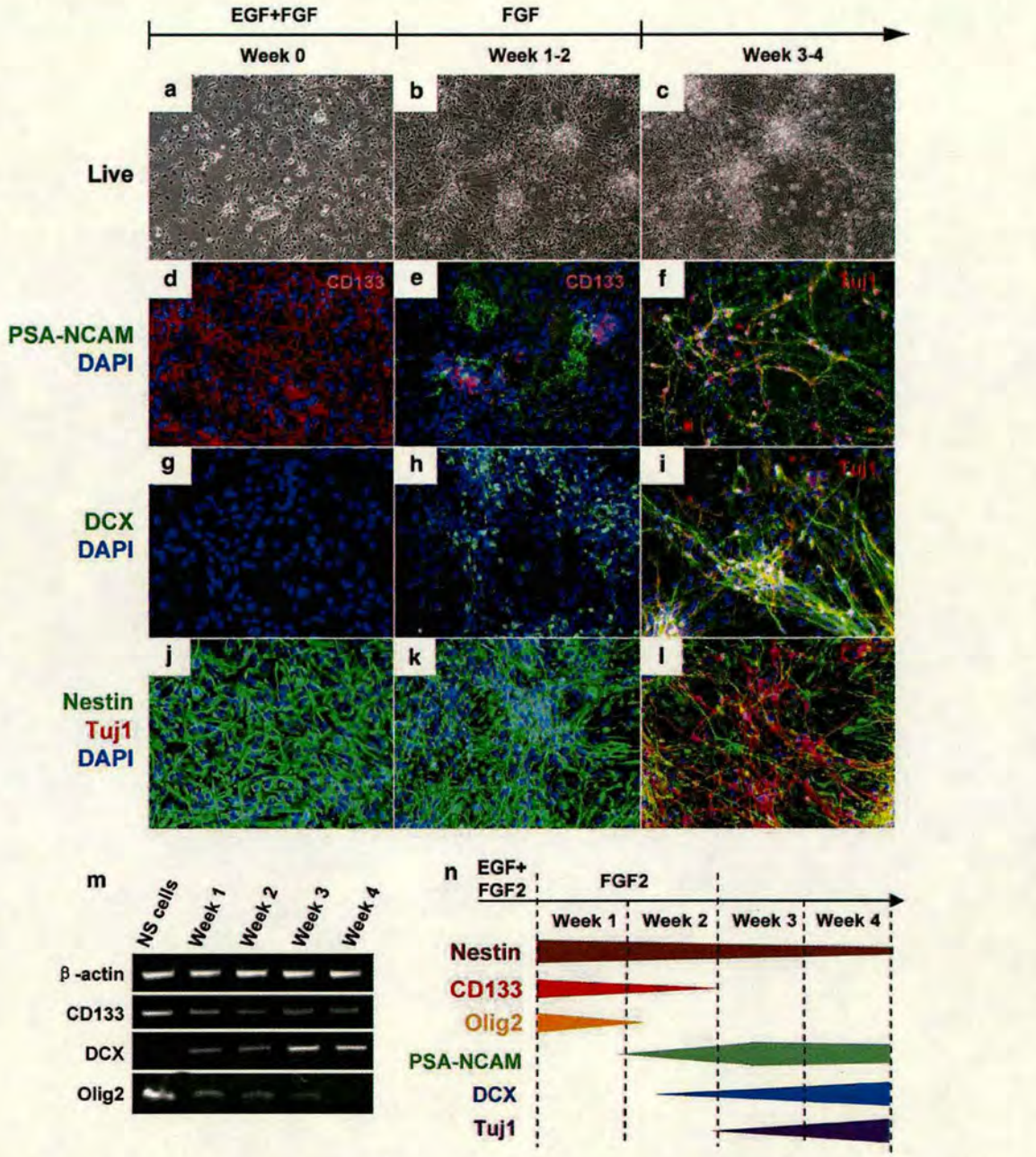

Fig. 6. Time-course of neuronal differentiation. Human NS cells undergo neuronal differentiation upon sequential withdrawal, first of EGF and then of FGF (a–m). The figure in n summarizes the qualitative marker changes over 4-week differentiation.

physiologically active, display underlying voltage-gated Na^+ conductance, and exhibit excitability, characteristic properties for mature nerve cells.

To clarify which type(s) of neurons human NS cells may be generated in culture, we stained derived neurons with different neuronal subtype markers. In both brain and spinal cord cell cultures, ~5% of Tuj1+ neurons displayed Calretinin expression (Fig. 8d, e). Tuj1+ neurons could be derived after adding Shh, FGF8, and/or B27 (either with or without retinol acetate) into differentiation medium, but to date we have not detected specific neuronal subtypes other than Calretinin expressing neurons from long-term expanded human NS cells (a small number of TH+ neurons could be generated from primary and non-clonal human NS cells, data not shown). The neuronal markers we have tested so far by antibody staining and RT-PCR include: Darpp32 Somatostatin, Parvalbumin, Calbindin, Neuropeptide Y, GAD67, GABA, Islet-1, TH, and ChAT.

Human NS cells can be genetically modified

A significant limitation of neurosphere cultures is the inefficiency of genetic labelling or manipulation. We transfected ~2.5×10^6 CB541 human NS cells with linearized pCAGGFPIP plasmid DNA by Nucleofection. Over 40% of viable cells

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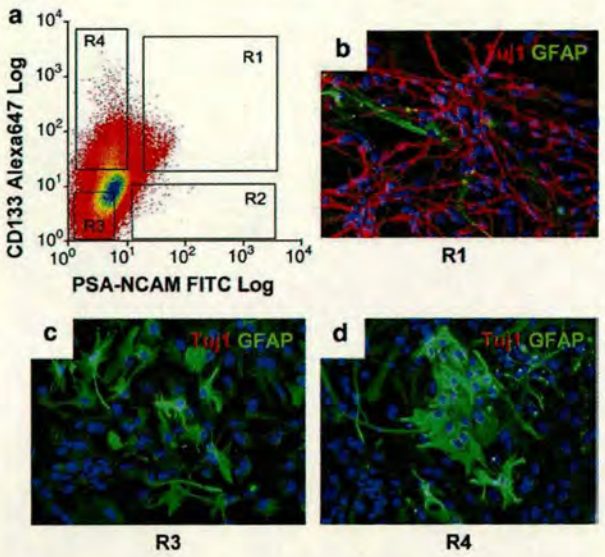

Fig. 7. Purified PSA-NCAM expressing cells generate neurons. On day 14 of neuronal differentiation (NS cells treated with FGF2 only), live cells were stained with anti-PSA-NCAM and CD133 antibodies and sorted using gates illustrated in a (R1–R4). Sorted cells were re-plated onto laminin coated dishes for further differentiation (without mitogen). Two weeks later, only PSA-NCAM+ cells (R1) generated Tuj1+ neurons, although the culture still contained GFAP positive cells (b) (Cells from R2 did not survive well after re-plating, probably due to limited number of cells). Most PSA-NCAM negative cells (R3 and R4) differentiated into astrocytes with high level of GFAP expression (c, d).

displayed Green Fluorescent Protein (GFP) expression 30 h after transfection. Human NS cells with stable and readily visualised GFP expression were established by repeated cell sorting at 3, 6, and 9 weeks after transfection. After the third sort, cells were homogeneously GFP positive and could be expanded for at least

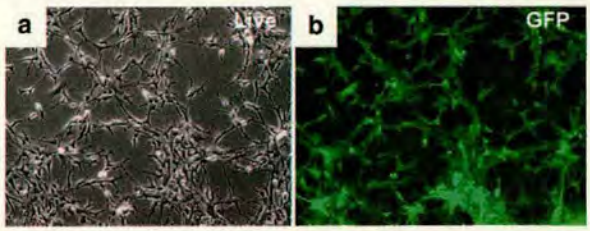

Fig. 9. Genetic modification of human NS cells. CB541 human NS cell line was transfected with pCAGGFPIP plasmid DNA. GFP+ cells were purified by FACS and expanded for > 3 months with retention of GFP expression (a, b).

3 months with retention of GFP expression (Fig. 9a, b). They also exhibited GFP expression after differentiation into neurons or glia (data not shown).

Discussion

Cultured neural stem cells hold considerable promise both in biological research and for potential cell-replacement therapies. Current studies of human neural cells largely rely on primary neurosphere cultures or genetic immortalization. However, the nature of cellular heterogeneity in neurospheres prevents direct interrogation of sphere-forming cell populations (Suslov et al., 2002; Reynolds and Rietze, 2005). It has been shown that committed progenitors can also generate sphere-like aggregates (Seaberg and van der Kooy, 2002), and that the size, number, and variable cellular compositions of neurospheres may not accurately reflect the proliferation activity, self-renewal capacity and developmental potential of the original sphere-forming cells (Singec et al., 2006). Thus, even if neurospheres are generated in clonal conditions, one cannot definitively infer the existence of neural stem cells in individual neurospheres (Reynolds and Rietze, 2005). Genetic immortalization allows extensive expansion and clonal analysis of cultured human neural progenitors (Flax et al., 1998; Villa et al., 2000). However, a major concern of perpetuated cells is that

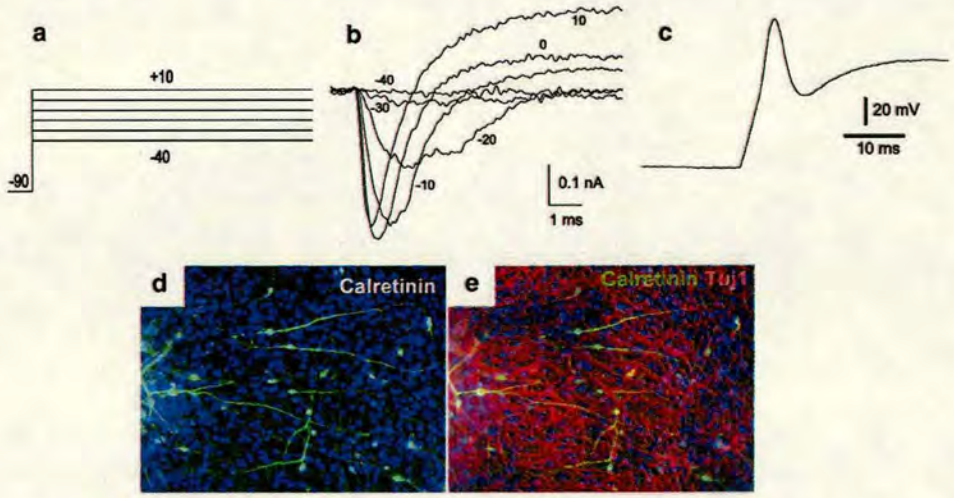

Fig. 8. Human NS cells generate mature neurons. Human NS cells generate mature neurons defined by electrophysiological attributes. In voltage-clamp recordings, depolarizing voltage steps between −80 and +10 mV from a holding potential of −90 mV (a). An example of current traces is presented in (b). Voltage response from the same cell is presented in (c). Cells were stimulated with a rectangular current pulse of 80 pA amplitude. Immunostaining show that ~5% of Tuj1+ neurons express interneuron marker Calretinin after 3–4 weeks differentiation (d, e).

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oncogenes may subvert normal regulation of cell cycle, apoptosis, and differentiation, so that perpetuated cells may not reliably reflect fully the properties of original cell populations. In addition, continuous oncogene expression in immortalized cells prohibits the use of these cells for cell therapy.

In this manuscript, we described adherent human foetal NS cells derived from both brain and spinal cord that 1) express appropriate markers of neural stem cells; 2) show clonogenicity and long-term stability without genetic immortalization; 3) exhibit tripotent differentiation potential, generating oligodendrocyte, astrocytes, and mature neurons even after prolonged expansion; and 4) are readily derived, expanded, and genetically manipulated in fully defined culture conditions. These findings establish that human NS cell lines are self-renewing and tripotent, human neural stem cell population *in vitro*.

Human NS cells display similar culture requirements to mouse NS cells (Conti et al., 2005; Pollard et al., 2006a,b). They propagate in the presence of EGF and FGF2, under which conditions differentiation into neurons and glia is fully suppressed. Both human and mouse NS cells can be expanded with addition of EGF only (Pollard et al., 2006b), suggesting that EGF is the major mitogen for NS cell self-renewal, although a contribution of autocrine FGF2 is not excluded. However, neither human nor mouse primary cells produce stable cell lines unless they are exposed to FGF2 during the first 2–4 weeks after plating. A possible contributing factor is that FGF2 may induce EGF responsiveness in NS cells in the first instance, which would be consistent with previous observations on other rodent neural progenitors (Ciccolini and Svendsen, 1998; Santa-Olalla and Covarrubias, 1999).

We find that laminin substrate is crucial for mouse/human NS cell neuronal differentiation (Conti et al., 2005) and optimal for human NS cell propagation. This indicates that laminin may play important roles in regulating neural cell behaviour. Indeed, mammalian laminin has been shown to present in neural stem cell niches including embryonic periventricular regions (Liesi, 1985) and the adult SVZ (Mercier et al., 2002). Rodent models with laminin receptor mutations display cortical abnormalities in association with pathological features of CNS diseases (Colognato et al., 2005).

Our data indicate that LIF is not required for the long-term stability of human NS cells. Human NS cells derived and expanded without LIF did not show cell crisis or senescence. This suggests the species specific effects of LIF reported for human neurosphere cultures could be indirect. It is possible that LIF may induce glial differentiation of progenitors (Bonni et al., 1997; Nakashima et al., 1999) that subsequently produce factor(s) that feedback on the progenitors to promote cell proliferation.

Human and mouse NS cells exhibit similar expression of transcription factors. They express neural precursor markers including Nestin, Vimentin, Sox2, Pax6, and Olig2; radial glia markers 3CB2, BLBP, and Glast; and regional markers Otx2 and Hoxb9 (Conti et al., 2005; Pollard et al., 2006a,b; and data not shown). We speculate that NS cells may represent a generic mammalian neural stem cell population *in vitro*. Although they exhibit some features of radial glia, it should be considered that the artificial nature of culture environments may result in unique cell populations *in vitro*. Therefore, NS cells do not necessarily have direct *in vivo* counterparts. In fact, the combination of transcription factor expression in mouse NS cells is not routinely observed during normal development (Pollard et al., 2006b).

Although human NS cells are highly expandable, their telomeric length shortens during expansion due to the absence of telomerase, which is consistent with earlier studies on human neurospheres (Ostenfeld et al., 2000). This implies human NS cells may undergo replicative ageing like most other somatic cells (Allsopp et al., 1992). However, recent studies have noted that progressive telomeric shortening is not necessarily a constant function of cell division. For example, several growth factors, including FGF2, may maintain telomeres of human stem cells without upregulation of telomerase activity (Yanada et al., 2006). In addition, it was reported that telomere shortening may not correlate with a cell's replicative history in a straightforward or quantitative manner (Hodes, 1999; Villa et al., 2004). Further studies are required to determine whether telomere length stabilises or shortens upon more extended human NS cell cultures.

We demonstrate that human NS cells are amenable to genetic modification and that their response to pharmacological agents or recombinant proteins could be directly inspected by videomicroscopy and immunostaining. These characteristics enable NS cells to be applied as a model system for both developmental and pharmaceutical research. In fact, mouse NS cells have been utilized in studies on cell reprogramming (Blelloch et al., 2006; Silva et al., 2006) to investigate epigenetic restrictions and commitment. Our data also show that human NS cells generate mature and functional neurons *in vitro*, defined by both marker expression and electrophysiological attributes. Although it seems that human NS cells have limited capacity to produce diverse neuronal subtypes *in vitro*, it is likely that particular culture conditions or glial support is required for generation of specific neuronal types. PSA-NCAM is a cell surface marker that was identified in human neural progenitor cells derived from embryoid bodies (Zhang et al., 2001). Here, we demonstrated that in the presence of EGF and FGF2, PSA-NCAM expression is suppressed in NS cells, and is up-regulated in early (FGF2 cultured) neuronal progenitor cells. Isolating PSA-NCAM expressing neuronal progenitors could enable more detailed characterization of early stages of neuronal differentiation *in vitro*. This strategy may also be applied to enrich for viable neuronal progenitors for potential neuroregenerative medicine.

In conclusion, human foetal NS cell lines represent a genetically normal self-renewing and tripotent human neural stem cell population *in vitro*. They can be applied to investigate fundamental questions in stem cell and developmental neurobiology. They may also serve as a scaleable source of human neurons and glia cells for genetic and pharmaceutical screening and possibly for cell-replacement therapies. It will be important to determine NS cell differentiation capacity to generate different neuronal subtypes *in vitro* and *in vivo*, and whether or how they may contribute to functional reconstruction in disease models. It should also be informative to investigate the relatedness of human NS cells to brain tumour initiating cells (Singh et al., 2004). Finally, it will be interesting to compare foetal NS cells with neural precursor cells derived from human ES cells (Conti et al., 2005) on the basis of long-term and clonal propagation.

Experimental Methods

Cell culture and time-lapse videomicroscopy

All studies with human tissue were performed under Ethical Approval from the Lothian Healthcare Trust using tissue donated with informed consent after elective termination of pregnancy. Human foetal brain (mainly

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cortex) and spinal cord tissue at embryonic 50–55 days (Carnegie stage 19–22) were carefully dissected in Neurobasal medium (Invitrogen) and dissociated into single cell suspensions with Accutase (Sigma) treatment. Primary cells were then plated onto laminin (10 mg/L, Sigma) coated dishes (Iwaki) in expansion medium comprising Euromed-N medium (formerly NS-A, Euroclone, Italy) or RHB-A medium (Stem Cell Scineces Ltd., UK), L-glutamine (2 mM final, Invitrogen), modified N2 supplement (Ying and Smith, 2003), B27 (20 ml/L final, Invitrogen), penicillin-streptomycin (10 ml/L final, Sigma), recombinant mouse EGF (10 ng/ml final, Peprotech), and recombinant human FGF-2 (10 ng/ml, Peprotech). To eliminate neurons in primary cultures, cells were transferred onto 0.1% gelatin (Sigma) coated dishes for 7 days before being re-plated onto laminin for further expansion. Medium was changed every 2 days, and cells were split 1:2 to 1:3 once cultures became confluent. Clonal human NS cell lines were generated by deposition of single cells into laminin coated 96-well plates using a Dako Cytomation MoFlo cell sorter, followed by continuous expansion. Medium was renewed by 50% change every 3 days in clonal assay, and cells were passaged once a month.

For neuronal differentiation, $\sim 1 \times 10^5$ human NS cells were plated into poly-ornithine (Sigma) and laminin coated 35-mm dishes in expansion medium without adding EGF. After 10 days, medium was switched to the mix Euromed-N:Neurobasal (1:1), supplemented with N2 (0.5×), B27 (1×), and FGF2 (10 ng/ml). Four days later, FGF2 was withdrawn from the medium, and after another 4 days, medium was switched to Neurobasal media supplemented with B27 (1×) and brain derived neurotrophic factor (BDNF, 20 ng/ml, R&D Systems). Differentiated cells were maintained up to 8 weeks after FGF2 withdrawal for electrophysiological studies. To derive oligodendrocytes, human NS cells were plated onto poly-ornithine/laminin coated plastic in expansion medium for 24–48 h. Medium was then changed to DMEM/F12 medium supplemented with N2 (1×), forskolin (10 nM), FGF2 (10 ng/ml) and PDGF (10 ng/ml, R&D Systems) for 14 days. From day 15, medium was switched to DMEM/F12 supplemented with N2 (1×), T3 (30 ng/ml), ascorbic acid (200 μM), and PDGF (10 ng/ml). PDGF was withdrawn from culture on day 22 to allow maturation. O4 positive could be detected after a total 5-week differentiation culture. For astrocyte generation, human NS cells were treated with 5% serum or 10 ng/ml BMP4 (R&D Systems) without mitogen for 2–3 weeks.

We prepared growth curves and time-lapse movies using IncuCyte™ imaging system with built-in software (Essen Instruments).

Immunostaining and RT-PCR

We used Alexa-Fluor secondary conjugates (Invitrogen) and DAPI (Sigma) in immunostaining. Primary antibodies were used at the following dilutions: Nestin (1:500; R&D Systems), Sox2 (1:400; Chemicon), GFAP (1:300; Millipore), Tuj1 (1:200, Covance), O4 (1:100, R&D Systems), 3CB2 (1:20, DSHB), BLBP (1:500, Abcam), Vimentin (1:20, DSHB), CD133 (1:10, Miltenyi Biotec), PSA-NCAM (1:200, Millipore), DCX (1:300, Cell Signalling), Neurofilament (1:300, Millipore), and Calretinin (1:200, Santa Cruz Biotech).

We used RNeasy kit (Qiagen) to extract total RNA and Superscript III (Invitrogen) to prepare cDNA. RT-PCR was performed for 30 cycles for all markers except β-actin for 25 cycles (denaturing for 40 s at 94 °C; annealing for 40 s at 56 °C, extension for 60 s at 72 °C). PCR products were resolved on 1.5% agarose gel. Primer sequences and product sizes are listed in Table 1.

Western blots

Cell extracts (50 μl) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% NuPage gel, Invitrogen) and transferred to a polyvinylidene difluoride membrane (Amersham). The membrane was incubated with 5% nonfat dry milk at 4 °C overnight and then probed with the respective antibody (anti-αtubulin, Abcam, 1:2000; anti-S100β, Santa Cruz Biotech, 1:1000). After three washes, the membrane was incubated with 1:5000 dilution of the appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature. Washed blots were subjected to enhanced chemiluminescence.

Telomerase activity, telomeric length, and chromosome analyses

Non-radioactive analyses for telomerase activity and telomeric length were performed using appropriate kits with supplied protocols (Roche). For karyotype assay, NS cells were treated with 5 ml 0.56% KCl for 15 min, fixed in acetic acid:methanol (1:3), spread onto glass slides, and stained with Giemsa solution.

Table 1
Primers used for RT-PCR

Gene	Forward primer	Reverse primer	Size (bp)
β-actin	GTC TTC CCC TCC ATC GTG	AGG TGT GGT GCC AGA TTT TC	181
BLBP	CCA GCT GGG AGA AGA GTT TG	CTC ATA GTG GCG AAC AGC AA	196
Calretinin	GCT CCA GGA ATA CAC CCA AA	CAG CTC ATG CTC GTC AAT GT	208
cd133	CAG AGT ACA ACG CCA AAC CA	AAA TCA CGA TGA GGG TCA GC	245
cd44	GGC TTT CAA TAG CAC CTT GC	ACA CCC CTG TGT TGT TTG CT-3	152
DARPP32	CAG AGG AGG AGG ATG AGC TG	GGT CTT CCA CTT GGT CCT CA	249
DCX	GAC AGC CCA CTC TTT TGA GC	TGG GTT TCC CTT CAT GAC TC	229
dlx1	TAC AGC TCA GCC TCG TCC TT	ACT TGG ATC GCT TGT TTT GG	179
GAD67	ATC GCT CCA CCA AGG TAC TG	ATT CGC CAG CTA AAC CAA TG	213
Gfap	GAA GCT CCA GGA TGA AAC CA	ACC TCC TCC TCG TGG ATC TT	165
Gfap-α	ACA TCG AGA TCG CCA CCT AC	ATC TCC ACG GTC TTC ACC AC	166
Gfap-δ	ACA TCG AGA TCG CCA CCT AC	CGG CGT TCC ATT TAC AAT CT	203
Glast	CTC ACA GTC ACC GCT GTC AT	CCA TCT TCC CTG ATG CCT TA	202
Hoxb9	TAA TCA AAG ACC CGG CTA CG	CTA CGG TCC CTG GTG AGG TA	198
Neuropeptide Y	CGC TGC GAC ACT ACA TCA AC	TAG GAA AAG GCC AGA GAG CA	177
olig2	CAG AAG CGC TGA TGG TCA TA	TCG GCA GTT TTG GGT TAT TC	208
otx2	AGA GGA GGT GGC ACT GAA AA	ATT GGC CAC TTG TTC CAC TC	188
Parvalbumin	GCG GAT GAT GTG AAG AAG GT	GCA GAG AGG TGG AAG ACC AG	249
pax6	GGG CAA TCG GTG GTA GTA AA	CTA GCC AGG TTG CGA AGA AC	190
Somatostatin	CCC AGA CTC CGT CAG TTT CT	CCA TAG CCG GGT TTG AGT TA	205
sox1	AAT CCC CTC TCA GAC GGT G	TTG ATG CAT TTT GGG GGT AT	224
sox2	GCC GAG TGG AAA CTT TTG TCG	GCA GCG TGT ACT TAT CCT TCT T	154
sox3	CCA AGG AGT GAA TGG GAG AA	AGA TCA CGG CAG AAA TCA CC	248

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Patch clamp recordings

To determine the electrophysiological attributes of neurons from human NS cells, we prepared seals between electrodes and cells in a bath solution consisting of 155 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 3 mM KCl, and 10 mM HEPES/NaOH (pH 7.4). In both voltage-clamp and current-clamp recordings, the pipette filling solution contained 128 mM KCl, 10 mM NaCl, 11 mM EGTA, and 10 mM HEPES/KOH (pH 7.4). We used an Axopatch 200B amplifier (Axon Instruments) to set the whole-cell voltage-clamp recordings, and a Digidata 1322A A/D converter (Axon Instruments) to digitize ionic currents. Stimulation, acquisition, and data analysis were carried out using PCLAMP 9 (Axon Instruments) and ORIGIN (Microcal Software) platforms. In voltage-clamp recordings, capacitive and leak currents were reduced by analogue circuitry and removed by P/4 method. We used borosilicate glass to prepare patch pipettes with a final 2–4 MΩ resistance.

Stable transfection of human NS cell lines

We transfected passage 6 CB541 human NS cells with ScaI linearized pCAGGFPIP plasmid DNA (Clontech) using Nucleofector (Amaxa Biosystems, program A-033). GFP expressing cells were repeatedly sorted by FACS 3, 6, and 9 weeks after transfection. Isolated GFP positive cells at week 9 were expanded to establish a reporter NS cell line.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2008.02.014.

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Adherent Neural Stem (NS) Cells from Fetal and Adult Forebrain

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[1]Centre Development in Stem Cell Biology, Institute for Stem Cell Research, School of Biological Sciences, University of Edinburgh, King's Buildings, Edinburgh EH9 3JQ, UK and [2]Department of Pharmacological Sciences and Center of Excellence on Neurodegenerative Diseases, University of Milano, Milan, Italy

Stable in vitro propagation of central nervous system (CNS) stem cells would offer expanded opportunities to dissect basic molecular, cellular, and developmental processes and to model neurodegenerative disease. CNS stem cells could also provide a source of material for drug discovery assays and cell replacement therapies. We have recently reported the generation of adherent, symmetrically expandable, neural stem (NS) cell lines derived both from mouse and human embryonic stem cells and from fetal forebrain (Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A. 2005. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol 3(9):e283). These NS cells retain neuronal and glial differentiation potential after prolonged passaging and are transplantable. NS cells are likely to comprise the resident stem cell population within heterogeneous neurosphere cultures. Here we demonstrate that similar NS cell cultures can be established from the adult mouse brain. We also characterize the growth factor requirements for NS cell derivation and self-renewal. We discuss our current understanding of the relationship of NS cell lines to physiological progenitor cells of fetal and adult CNS.

Keywords: adult, EGF, FGF-2, mouse, neural, radial glia, stem cell

Introduction

A stem cell is an uncommitted cell that can divide repeatedly while maintaining potency to generate differentiated cell types. In contrast, cells that exhibit only a limited number of divisions before a change in potency or overt differentiation are termed progenitor cells. The functional distinction between stem cells and progenitors can only be defined by experimental determination of the capacity for self-renewal. Our current view of central nervous system (CNS) ontogeny supposes a founder population of cells capable of forming both neurons and glia in response to inductive patterning and differentiation cues conveyed by adjacent cells (Panchision and McKay 2002). This mechanism generates the appropriate diversity of neuronal and glial subtypes in the correct place and time. However, whether the cells that form the CNS in vivo are stem cells or transient progenitors is uncertain (Anderson 2001).

During brain development, neural progenitors are localized in the pseudostratified epithelium of the germinal zone surrounding the ventricles. Postmitotic neurons migrate away from this ventricular zone, guided by the elongated processes of radial glia that act as scaffolds in the maturing brain. It has recently become evident that within the developing cortex, and possibly other regions, radial glia not only fulfill this structural role but also are progenitor cells that divide to generate neuroblasts and neurons (Noctor and others 2001; Malatesta and others 2003; Anthony and others 2004). In the adult brain, ongoing neurogenesis has been convincingly documented in 2 regions, the subventricular zone (SVZ) of the lateral ventricles and the subgranular layer of the hippocampus (Gage and others 1998; Doetsch and others 1999). Cells extracted from the germinative zones of the developing and adult nervous system can be expanded in vitro (Temple 1989; Cattaneo and McKay 1990; Reynolds and others 1992; Reynolds and Weiss 1992). These findings have raised hopes that in the future it may be possible to stimulate endogenous CNS repair mechanisms or to replace dysfunctional or dead cells with cells generated in vitro. However, these remain ambitious and challenging goals (Lindvall and others 2004).

An ability to continuously expand stem cells clonally by symmetrical division offers critical opportunities for experimentation, allowing identification of factors and genes acting directly on the stem cells to regulate self-renewal and commitment (Smith 2001; Gottlieb 2002). Clonal and adherent expansion also enables efficient genome engineering and genetic screening, as established for embryonic stem (ES) cells (Bradley and others 1992). Derivation of adherent neural stem (NS) cell lines with stable neuronal differentiation potential has recently been described (Conti and others 2005). These have been termed NS cells to highlight the similar experimental attributes compared with ES cells.

Initially, NS cells were derived from ES cells differentiated into heterogeneous neuroepithelial progenitors in adherent monolayer culture (Ying and others 2003). Fibroblast growth factor (FGF-2) plus epidermal growth factor (EGF) supported expansion of a distinct subset of progenitor cells attached to tissue culture plastic in a defined basal media. These cells lost the heterogeneous morphology and marker expression of the primary neuroepithelial population (Li and others 1998; Ying and others 2003). Instead, over several passages, the cultures acquired a homogeneous morphology and were shown to uniformly express nestin and Sox2, although extinguishing the expression of Sox1. These cells are stem cells as they are clonogenic and maintain indefinitely the capacity to generate both neurons and astrocytes. NS cells display many hallmarks of radial glia by morphology and molecular markers, including brain lipid binding protein (BLBP), RC2, GLAST, and Pax6 (Gotz 2003; Rakic 2003). Thus, neural differentiation of ES cells initially entails conversion to a transient Sox1-positive panneural progenitor cell population, containing a subpopulation of cells that mature to a specific Sox1-negative state without differentiation and may then be stably maintained and expanded using EGF plus FGF-2 (Fig. 1).

Similar NS cell lines were also isolated from fetal brain tissue. Either primary cell cultures or long-term expanded neurospheres allowed to settle onto gelatin-coated flasks and left

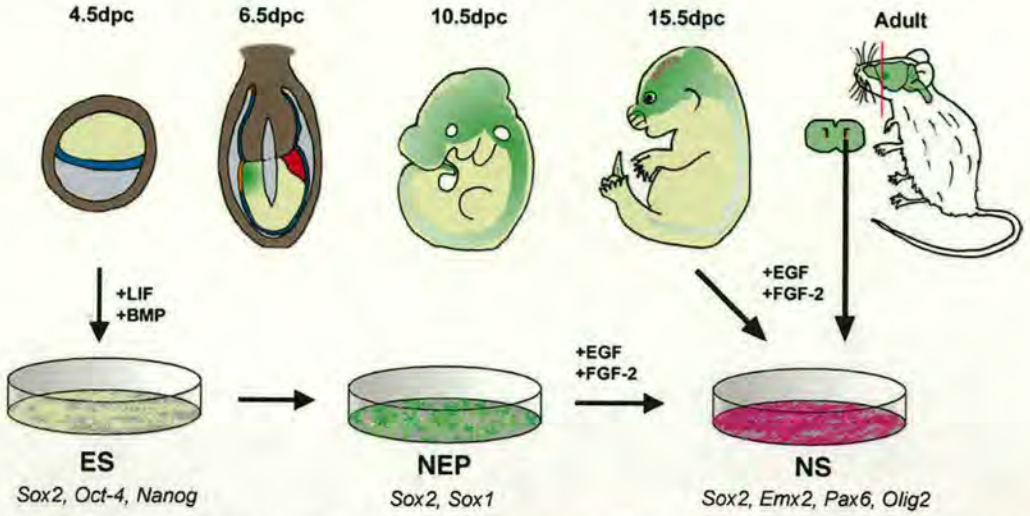

Figure 1. ES cells, neuroepithelial progenitors (NEPs), NS cells, and their in vivo sources. ES cells can be derived from and have similarities to the epiblast of late blastocyst stage embryos (left). ES cells can be readily converted to transient Sox1+ neuroepithelial cells (green) analogous to E8.5–10.5 neural tube. Some Sox1+ progenitors progress to Sox1–, Sox2+ progenitor cells that can be expanded as stem cell lines (NS cells) by the action of EGF and FGF-2 (middle/right). NS cells have similarities to forebrain radial glia and can be derived from fetal forebrain. In this report, we show that NS cells can also be derived from the SVZ of the lateral ventricle of adult mouse forebrain

undisturbed in EGF plus FGF-2 will reproducibly outgrow NS cells that are indistinguishable in all essential features from those derived from ES cells (Conti and others 2005). Our observations suggest that such cells, rather than being differentiation products of the neurosphere (Gregg and Weiss 2003), are in fact the resident stem cells. Indeed, NS cells will readily form neurospheres if detached from a culture substrate. The finding that the complexity of neurosphere cultures, which contain a mixture of stem cells, committed progenitors, and differentiated cells (Suslov and others 2002; Bez and others 2003), can be eliminated by adherent culture parallels observations with ES cells. In adherent culture, ES cells self-renew with minimal differentiation (Smith 2001), whereas suspension culture induces aggregation and differentiation into embryoid bodies (Martin and Evans 1975; Doetschman and others 1985).

In this report, we investigate whether cells capable of giving rise to NS cell cultures are restricted to developmental stages or may also be present in the adult brain. Further, we examine the requirement for EGF and FGF-2 in the derivation and maintenance of NS cells. We discuss how these data, together with other recently published findings, are shedding light on the nature of NS cells and their relationship to endogenous cell types.

Materials and Methods

NS Cell Derivation and Culture

The following protocol was used to derive the adult NS cell line ANS-1. A 2-month-old wild-type (CD1 strain) mouse was sacrificed, and an area encompassing the SVZ surrounding the lateral wall of the forebrain ventricle was dissected. Tissue was dissociated with accutase (Sigma, St. Louis, MO) for 5 min at 37 °C and mechanically dissociated using a fire-polished glass Pasteur pipette. Cells were washed once in phosphate-buffered saline (PBS) and then plated onto a 6-well untreated plastic plate (Iwaki, Iwaki, Japan) in NS-A media (Euroclone, Milan, Italy) plus N2 supplement and 10 ng/mL of both FGF-2 and EGF (Peprotech, Rocky Hill, NJ). This medium is termed expansion medium. These cultures were maintained for 3 weeks, with a half volume of medium exchanged every week. Small spheres formed within the period and were collected by centrifugation at 700 rpm for 30 s. The pellet was resuspended in accutase and cells dissociated by repeated pipetting. Cells were cultured in expansion media for a further 10 days. Upon replating of the newly formed spheres into a fresh T25 flask, attachment and outgrowth occurred within 4–5 days. These cells showed typical morphology of NS cells. They were subsequently expanded in these adherent conditions. Cells underwent rapid expansion and at 70% confluence were passaged 1:3 to 1:5 every 3–4 days for more than 35 passages to date. A procedure identical in all essential features was followed to derive a second adult NS cell line from a female mouse on a mixed 129 × MF1 background.

At higher densities and at earlier passages, NS cells can tend to aggregate and spontaneously form neurospheres. This may be reduced substantially through plating onto a tissue culture plastic precoated with gelatin (15 min treatment of 0.1% solution) or effectively eliminated by culture on poly-ornithine/laminin (30- to 60-min treatment with poly-ornithine solution 0.01%; wash twice in PBS and then add a 10-μg/mL laminin solution for at least 2 h) (Sigma). Detailed protocols for routine handling of NS cell lines are available elsewhere (Conti and others 2005).

Characterization of NS Cells

NS cells were induced to differentiate after replating 0.5–1 × 10^5 cells onto a laminin-coated treated 4-well plate. For astrocyte induction, cells were exposed to NS-A media supplemented with N2 (in-house preparation) and 1% fetal calf serum (Sigma) or 10 ng/mL of bone morphogenetic protein (BMP)-4 (R and D Systems, Minneapolis, MN) for 7–12 days. A minor fraction of adult-derived NS cells differentiated into neurons in these conditions. For more efficient neuronal induction, cells were treated for 5–7 days with NS-A media supplemented with B27 (Invitrogen, Carlsbad, CA) plus 10 ng/mL FGF-2 (Peprotech) and then switched to NS-A:Neurobasal media (1:1) supplemented with B27, 0.5× N2, and no growth factors. For all differentiations, half the volume of medium was exchanged for fresh medium every 2–3 days.

To test the effect of culture in EGF only, NS cells lines were passaged at least 5 times prior to analysis in expansion media without FGF-2. Colony formation assays were performed by plating 10 000 cells into a 9-cm culture dish and scoring colonies after 7–10 days. The fibroblast growth factor receptor (FGFR) inhibitor SU5402 (Mohammadi and others 1997) was used at 5 μM (Calbiochem). For bromo-deoxyuridine (BrdU) assays, NS cells were plated on gelatin-coated 12-well plates with expansion media, incubated at 37 °C overnight for recovery and then cultured with 10 μM BrdU (Sigma) for 2 h. Cells were then fixed in 4% paraformaldehyde for 15 min and washed 3 times with PBS (Sigma). A total of 500 μL of 2 M HCl was added to each well at room temperature for 1 h followed by a further 3 PBS washes. Cells were then immunostained with anti-BrdU antibody (Sigma). Growth curves for EGF-independent lines were determined by plating 1000 NS cells in each well of gelatin-coated 6-well plates in expansion media with or without

FGF-2. Cells in duplicate plates were cultured for 7 days with medium changes every 2 days. Cells were counted by hemocytometer every 24 h after dissociation with accutase.

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA) and cDNA generated using Superscript III (Invitrogen). cDNA was normalized via real-time polymerase chain reaction (PCR) determination of β-actin level (Lightcycler, Roche, Branchburg, NJ). PCR was performed for 30 cycles for all markers except β-actin (25 cycles), using primers as described in Conti and others (2005). Antibodies and protocols for immunocytochemistry are described elsewhere (Conti and others 2005).

New Results and Discussion

Derivation and Characterization of Adult NS Cell Lines

NS cell lines derived from ES cells or from fetal forebrain share a range of features with radial glia (Conti and others 2005). Although radial glia have a finite existence, differentiating shortly after birth, they may be the source of long-lived adult CNS stem cells. Recent fate-mapping experiments have indicated that the type B stem cells in the adult SVZ derive from a subpopulation of fetal radial glia (Merkle and others 2004). Therefore, radial glia and adult SVZ astrocytes appear to comprise a continuous lineage with stem cell potential (Alvarez-Buylla and others 2001; Merkle and others 2004).

We were therefore interested in determining whether NS cell lines could be established from adult mouse SVZ. We derived primary neurospheres through dissection of the lateral forebrain ventricle and culture for 3 weeks in basal media supplemented with N2 and EGF plus FGF-2. These spheres readily attached when finely dissociated and replated in a minimal volume of media and left undisturbed for several days. Upon attachment, cells outgrew rapidly and after 7 days in culture were dissociated with accutase and replated onto fresh flasks. We find that in these conditions, cell cultures could be maintained subsequently as adherent cell lines with characteristic NS cell properties. We established cell lines from a male CD1 mouse and a female 129 × MF1 hybrid animal that we have termed ANS-1 and O4ANS, respectively.

Growth characteristics and morphology of both ANS-1 and O4ANS are similar to characterized ES- and fetal-derived NS cell lines (Conti and others 2005). Cells divide approximately every 30 h, and the population forms lattice/net structures at higher densities (Fig. 1*A*). ANS-1 has been expanded for over 30 passages without crisis. The adult NS lines do have a tendency, particularly during early passages and at higher densities, for cells to aggregate and detach from the plate, spontaneously reforming neurospheres. As described previously for fetal- and ES-derived NS cells, we find that NS cells can also be expanded on a laminin substrate whereupon aggregation and detachment are reduced and cells can be expanded to confluence, facilitating routine propagation.

Interestingly, these adult-derived NS cells appear to have reverted to a radial glia–like state. Immunocytochemical staining indicates uniform expression of the markers of radial glia and NS/neural progenitor status: RC2, nestin, and BLBP (Fig. 2*B–D*). Further, RT-PCR analysis and antibody staining identify expression of the transcription factors Sox2, Olig2, Mash-1 and at lower levels both Emx2 and Pax6 (Fig. 2*G*). The transit amplifying cells within the adult SVZ express Dlx2, which is downregulated upon exposure to EGF in vitro (Doetsch and others 2002). We do not detect transcripts for Dlx2 in ANS-1 or O4ANS by RT-PCR. Further, by both RT-PCR and immunocytochemistry, we find little or no GFAP expression in NS cells cultured on laminin (not shown). These data indicate that NS cells do not correspond exactly to either transit amplifying cells or endogenous GFAP-positive stem cells. It is possible that they derive from the former, however, as has been described for neurosphere formation (Doetsch and others 2002).

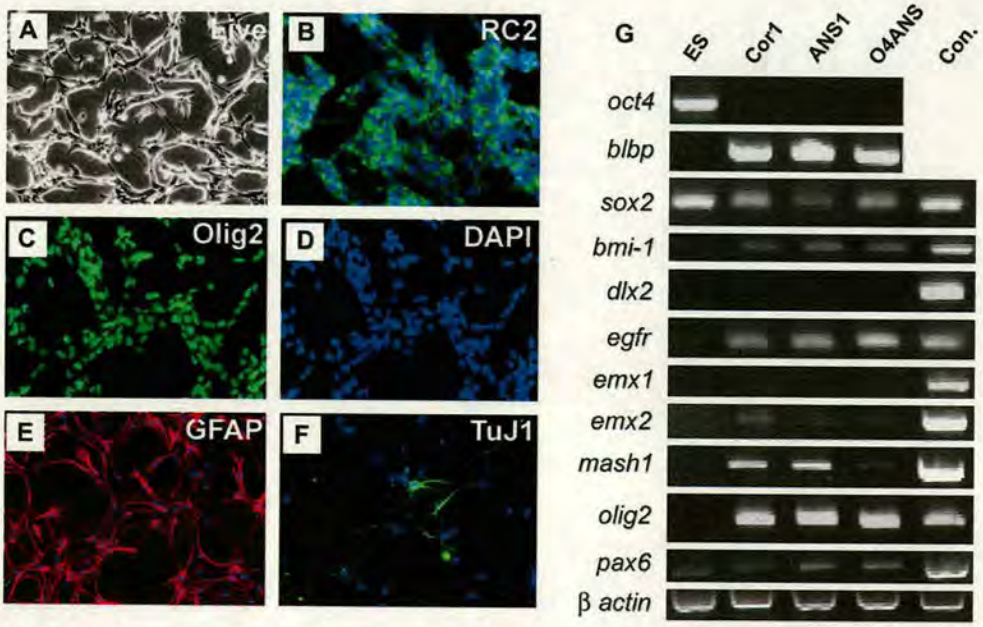

Figure 2. NS cells derived from adult SVZ are similar to fetal forebrain-derived NS cells. ANS-1 cells proliferate as an adherent monolayer and have morphological characteristics similar to ES cell–derived and fetal-derived NS cells (*A*). All cells express RC2 (*B*) and Olig2 (*C*) (corresponding DAPI, *D*) and can differentiate into astrocytes (*E*) and neurons (*F*) at passage 30. By RT-PCR, adult SVZ-derived NS cells, ANS-1, and O4ANS express mRNA for transcription factors and radial glia markers observed in the fetal line, Cor1 (*G*). Positive control is E12.5 + E16.5 embryonic head (Con).

Upon differentiation through exposure to serum on a laminin substrate, we find that the adult NS cells are capable of differentiating into neurons and astrocytes (Fig. 2*E,F*). Differentiation is also induced by treatment with BMP4, possibly reflecting its endogenous role as a paracrine factor released by ependymal cells that promote astrocyte fate (Lim and others 2000). Together, these results suggest that adult SVZ cells isolated and expanded with EGF and FGF-2 can be maintained as adherent stem cell lines in vitro with characteristic markers of NS cells. We have also analyzed a set of NS cell marker genes, identified through an Affymetrix oligonuclelotide microarray approach, and find common expression between adult-, fetal-, and ES cell–derived NS cell lines (data not shown).

A set of master transcription factors expressed by NS cells may confer capacity for self-renewal in the context of a permissive epigenome. These transcription factors may not coexist in any in vivo progenitor (see below) but reflect the in vitro environment and growth factor stimulation. Sox2 is expressed in most cell types within the adult SVZ niche and may be required to maintain neural progenitors in an undifferentiated proliferative state during development (Ellis and others 2004; Pevny and Placzek 2005). Mash-1 has recently been shown to mark the adult forebrain SVZ transit amplifying cell population (Parras and others 2004). Furthermore, Emx2 has been ascribed a role in maintaining multipotent neural progenitors (Heins and others 2001). Both Pax6 and Olig2 are expressed within the adult SVZ progenitor domain (Hack and others 2005; Kohwi and others 2005). Olig2 expression has also been associated with amplification of gliogenic progenitors derived from ES cell differentiation (Xian and Gottlieb 2004). Interestingly, several of these transcription factors are known to cross-regulate and corepress one another (Schuurmans and Guillemot 2002). The combination of FGF-2 plus EGF may create a synthetic cell state with an appropriate balance of these key transcription factors to suppress lineage commitment and allow self-maintaining divisions, analogous to the ES cell state (Smith 2001).

Derivation of NS Cell Lines Requires FGF Together with EGF but Does Not Depend on Neurosphere Formation

As described above, NS cells have been established from adult and fetal tissues via an initial suspension culture/neurosphere step prior to plating down and expanding as adherent cell lines. This raises the question whether suspension culture may be a necessary event for formation of NS cells. Direct isolation and culture of neural progenitors in adherent conditions have been reported over many years by several investigators using FGF-2 for survival/expansion of the cells (Johe and others 1996). However, these cultures change their properties over time (Qian and others 2000). Such primary cultures should not be considered equivalent to expanded long-term stem cell lines.

Surprisingly, few studies have reported the application of EGF and FGF-2 in combination in adherent culture. Accordingly, we plated dissociated E12.5 cortex directly onto laminin in the presence of EGF and FGF-2 to determine whether NS cell lines could be established directly without intervening neurosphere formation. Cells readily attach in these conditions and rapidly acquire a homogenous morphology. In the presence of both growth factors, cells are uniformly immunoreactive for Olig2, RC2, BLBP, and nestin (Fig. 3). These cells behave similarly to previously characterized NS cell lines cultured on laminin. We have named a line of NS cells derived in this manner Cor2. Cor2 can be serially passaged with retention of ability to differentiate into neurons and astrocytes. We conclude that neurosphere formation is not necessary for derivation of fetal NS cells.

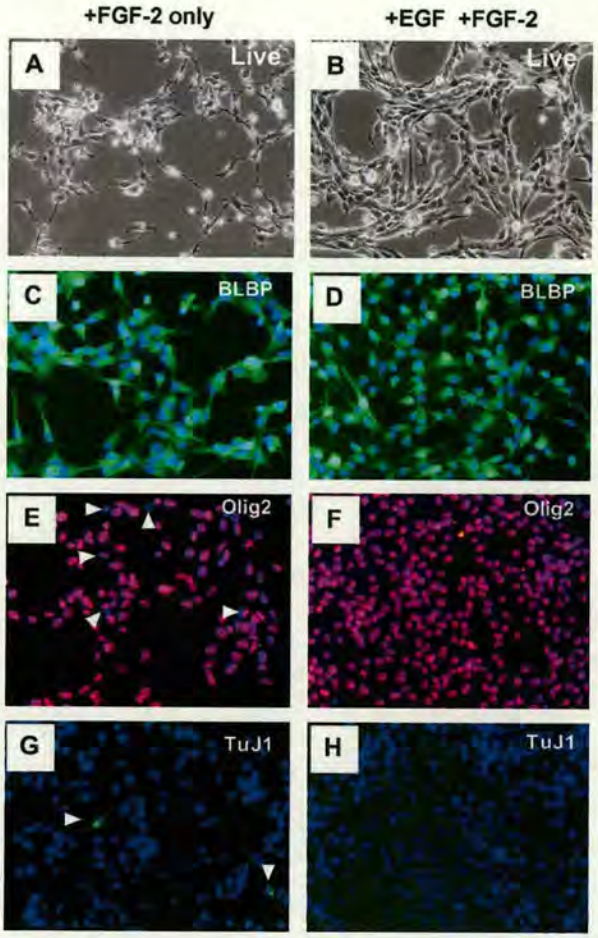

Figure 3. NS cell derivation requires both FGF and EGF but not neurosphere formation. E12.5 cortical progenitors were directly plated onto a laminin substrate without neurosphere formation. Cor2 was expanded in EGF and FGF-2 and resembles previously established NS cell lines by both morphology (*B*) and antibody staining (*D, F, H*). Cor2F was expanded in FGF-2 without EGF and has a more heterogeneous appearance (*A*). Olig2 and TuJ1 immunocytochemsitry also reveal heterogeneity and spontaneous neuronal differentiation for Cor2F (*E, G*, white arrowheads).

Previous studies have indicated that EGF alone is insufficient to generate embryonic neurospheres until late embryogenesis, whereas FGF-responsive cells are present earlier (Tropepe and others 1999). The distinction between early and late progenitor cells is thought to reside in levels of epidermal growth factor receptor (EGFR) that may relate to the developmental switch from a neurogenic to a gliogenic phase (Burrows and others 1997; Qian and others 2000; Sun and others 2005). We have been unable to isolate NS cells from ES cells using EGF or FGF-2 alone (Conti and others 2005). Here we tested whether either of these factors alone was sufficient to generate adherent NS cell lines from fetal cortex. Similar to previous studies, we found that EGF alone is not able to expand E12.5 progenitors and cultures rapidly die in suspension culture without attaching to the laminin substrate (not shown). In FGF-2 alone on a laminin substrate, we find that cells can be expanded through more than 10 passages and can be cryopreserved. We termed these cells Cor2F. Importantly, however, compared with cells expanded with EGF and FGF-2 in combination, FGF-2 cultures are more

heterogeneous by morphology; contain a proportion of immature neurons, identified by TuJ1 staining; divide more slowly; and at early passages, contain a relatively high incidence of dying cells (Fig. 3). It is possible that paracrine EGFR ligands can sustain stem cells in these conditions, but are in limiting supply.

Taken together, these results suggest that although FGF-2 is specifically required for initial derivation of NS cells, addition of EGF is important for expansion of homogenous NS cell cultures with efficient suppression of differentiation and apoptosis. FGF-2 may serve to induce EGFR expression in culture and/or to configure the epigenetic state of key regulatory loci such as *Sox* genes. There are data to support each of these possibilities; EGFR can be induced in culture by FGF-2 (Ciccolini and others 2005), and EGFR overexpression in early progenitors confers characteristics of late SVZ progenitors (Burrows and others 1997). Oligodendrocyte precursor cells can be converted to multipotent neurosphere-forming stem cells, and this is associated with modulation of chromatin at the *Sox2* locus leading to its reexpression (Kondo and Raff 2004). Interestingly, the initial requirement for FGF in setting up the NS cell state is reminiscent of its transient requirement in the reprogramming of primordial germ cells into pluripotent self-renewing embryonic germ cells (Matsui and others 1992; Resnick and others 1992).

EGF Alone Can Maintain NS Cell Self-Renewal

Established NS cells expanded in EGF/FGF-2 cannot be maintained in FGF-2 alone as removal of EGF from culture media results in massive cell death within 24 h (Conti and others 2005). Death can be avoided by provision of a laminin substrate, but FGF-2 is insufficient to maintain NS cells as passagable cell lines and cultures begin to undergo differentiation. This is more extreme than the situation described above for primary isolates in FGF-2 on laminin that can be continuously propagated, albeit with accompanying differentiation. The reason for this difference is unclear at present, but one possibility could be different levels of paracrine EGFR stimulation. The requirement for EGF-mediated signaling to maintain NS cell self-renewal may reflect endogenous neuregulin or transforming growth factor (TGF)α signals (Tropepe and others 1997; Schmid and others 2003; Ever and Gaiano 2005).

We investigated whether the converse was true, namely, can established NS cell lines be maintained with EGF alone upon withdrawal of FGF-2? We cultured ES cell-derived (CGR8-NS, NS5) and fetal-derived (Cor1, Cor1-3) NS cells in EGF alone. NS5 and Cor1-3 are clonally expanded cell lines. Surprisingly, withdrawal of FGF-2 did not result in any striking change in NS cell morphology or behavior (Fig. 4). NS cells withdrawn from FGF-2 maintain their homogenous morphology and marker staining, and differentiation to GFAP-expressing astrocytes or β-tubulin–expressing neurons remains fully suppressed (Fig. 4*J,K*). EGF-only propagated cells retain the capacity to generate astrocytes and neurons at a frequency similar to NS cells expanded in parallel in EGF plus FGF-2. Furthermore, we found that NS cells plated at clonal density formed colonies with similar frequency in EGF alone as in EGF plus FGF-2 (Fig. 4*G*).

The frequency of NS cell colony formation in either condition is around 1%. This low efficiency raises the possibility that there is a hierarchical organization within NS cell cultures, belying their homogeneity in marker expression. However, all colonies examined expand continuously similar to parental cells. Thus, there is no evidence for progenitors with finite proliferative lifespans, for example, as are observed in keratinocyte cultures (Barrandon and Green 1987). It seems more likely therefore that low colony-forming frequency is the result of culture stress at low density and/or dependence on autocrine stimulation rather than arising from heterogeneity of potency within the population.

In order to evaluate a potential role for autocrine FGF signaling in NS cell self-renewal, we employed a pharmacological inhibitor of FGFR (SU5402) (Mohammadi and others 1997). Addition of this inhibitor at a concentration (5 μM) effective at blocking FGFR signaling in other assays (Ying and others 2003) did not impair frequency of colony formation, although colonies were slightly smaller. SU5402 does impact upon cell population doubling time. EGF-only cultures show a lag phase in expansion after replating compared with parallel EGF plus FGF-2 cultures. BrdU pulse labeling indicates that the proportion of cells in S phase is similar in each condition, suggesting that the increased population doubling time may be due to higher cell death. In the presence of SU5402, the slower doubling rate of EGF-only cultures does not increase with cell density (Fig. 3*E–H*). These data suggest that autocrine FGF signaling can contribute to NS cell propagation. Therefore, more detailed analyses are required to determine whether there is an absolute requirement for FGF.

Are NS Cell Lines Equivalent to Forebrain Radial Glia?

A critical issue to consider when characterizing any stem/progenitor cell source in vitro is whether these cells have an in vivo counterpart and which cell type is the cell of origin (Doetsch and others 2002; Buehr and others 2003; Joseph and Morrison 2005). NS cells isolated from all sources express markers of radial glia (RC2, GLAST, BLBP, vimentin). Radial glia emerge during mouse development from Sox1-expressing neuroepithelial cells. In vivo fate-mapping studies along with characterization of purified radial glia in vitro have revealed a function for these cells not only as scaffold/guides for migrating neuroblasts (Rakic 1971) but also as proliferative founders of neuronal and, later, astrocyte, oligodendrocyte, and ependymal cell types (Alvarez-Buylla and others 2001; Gotz 2003; Merkle and others 2004).

In vivo, radial glia are heterogeneous in terms of their transcription factor expression profile, likely associated with elaboration of positional cues (Kriegstein and Gotz 2003). However, we found that NS cell lines, derived from either ES cells or fetal forebrain (cortex or striatum), express Pax6 and Emx2, although being negative for ventral markers such as Lhx6 (Schuurmans and Guillemot 2002). Pax6 and Emx2 within cortical radial glia are present in forebrain where they are suggested to function in the maintenance of neuronal potential and symmetrical self-renewal, respectively (Heins and others 2001, 2002). An unresolved issue is whether radial glia from regions other than forebrain, such as midbrain and spinal cord, can give rise to NS lines and if so whether they retain any positional specification. Reports using neurospheres and adherent neural progenitor cultures have suggested a respecification of positional markers when cells are exposed to growth factors in vitro (Gabay and others 2003; Santa-Olalla and others 2003; Hack and others 2004).

So are NS cells equivalent to fetal cortical radial glia? Expression of Pax6 and Emx2 by NS cells is consistent with a dorsal forebrain identity, but Mash-1 and Olig2 are found ventrally within the developing forebrain. There may exist within the developing CNS a rare cell population that coexpresses all

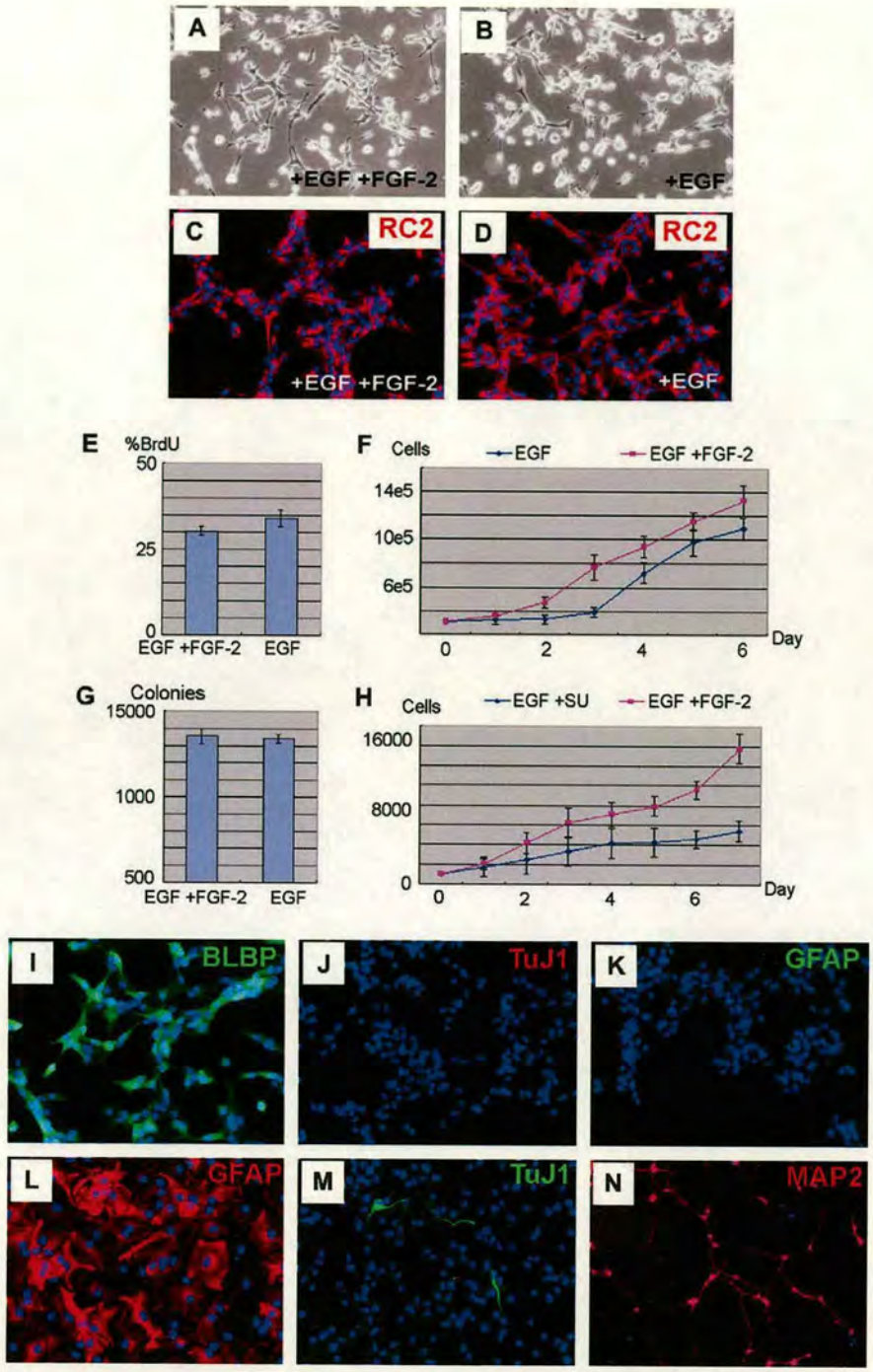

Figure 4. Addition of EGF is sufficient to maintain NS cell self-renewal. CGR8-NS cells (passage 19) were expanded for at least a further 5 passages with EGF alone. Compared with parallel cultures expanded with EGF plus FGF-2, we find no dramatic change in cell morphology (*A*, *B*) or radial glia marker RC2 (*C*, *D*). BrdU incorporation assays (*E*). Growth curves for CGR8-NS cells in EGF compared with EGF/FGF-2 (*F*) and in the presence of FGFR inhibitor (SU5402, 5 μM) (*H*). NS colony formation; 10 000 cells were plated and colonies scored after 7–10 days in culture (*G*). CGR8-NS cells expanded in EGF alone retain homogenous BLBP expression (*I*) but lack spontaneous differentiation to neurons (*J*) or astrocytes (*K*). Differentiation to astrocytes can occur to either astrocytes (upon serum treatment, *L*) or neurons (upon EGF withdrawal, *M*; or FGF-2 exposure and subsequent withdrawal, *N*).

these transcription factors. However, it is equally likely that this situation might reflect a despecification of progenitors when exposed to in vitro conditions. Recent studies have described the activation of Olig2 in culture through the action of FGF-2 and a relaxation of in vivo fate restriction (Chandran and others 2003; Gabay and others 2003; Hack and others 2004). By real-time quantitative RT-PCR, we find that Olig2 is upregulated in NS cells in the presence of FGF-2. Levels of Olig2 mRNA are around 5-fold higher in NS cells grown with EGF and FGF-2 compared with EGF alone in 3 different NS cell lines (not shown). These results highlight the artificial nature of cell culture, emphasizing the need for caution in extrapolation of in

vitro findings to normal development or physiology without parallel in vivo data.

Appreciation of the positional identity present within NS cell lines is invaluable for designing strategies to generate the desired neuronal subtypes. For cell-based pharmaceutical screening or transplantation to be viable, the critical factor is access to an unlimited stem cell resource with the potency to generate a range of cell types of the mature CNS. A range of neuronal subtypes can be generated through differentiation of ES cells, using opportunistic protocols or conditions designed to mimic developmental inductive events (Kim and others 2002; Wichterle and others 2002; Barberi and others 2003). NS cells and neurospheres generate largely γ-aminobutyric acidergic neurons, whereas ES cell–derived radial glia precursors generate glutamatergic neurons if they are not exposed to EGF/FGF-2 (Bibel and others 2004). EGF/FGF-2 may act to induce dominant ventral specification factors, such as Mash-1, that determine neuronal phenotype. For NS cells, it remains to be seen if environmental signals may promote diverse neuronal subtype differentiation. Genetic fate-mapping studies of radial glia suggest that these cells serve as founders for a range of neuronal subtypes within the nervous system, although this remains controversial (Anthony and others 2004; Gotz and Barde 2005). Therefore, a key question is whether the NS cell positional values established in vitro through the action of EGF and FGF-2 can be overridden by other extrinsic signals. Alternatively, do mitogens exist that allow capture and expansion of NS cells similar to NS cells but with distinct regional specification reflected in different repertoires of key transcriptional regulators?

Conclusions

Here and elsewhere, we have reported the ability to isolate and expand adherent NS cell lines from ES cells, fetal forebrain, and adult forebrain (Fig. 1) (Conti and others 2005). NS cells are clonogenic, apparently homogenous, symmetrically expandable, adherent cultures. They thus constitute a pure stem cell resource with experimental advantages comparable with ES cells. Derivation of these NS cell lines requires FGF-2. However, once established, NS cell self-renewal can be sustained by exogenous EGF alone (Fig. 5). NS cells may be related to brain cancer stem cells (Singh and others 2004), notably those of aggressive gliomas resulting from activation of EGFR. Identification of functional regulators of NS cell self-renewal might therefore lead to novel therapeutic strategies to target cancer stem cells. Further investigations will determine whether the characteristics of NS cells represent a unique cell state acquired in vitro or mirror features of rare endogenous CNS progenitor cells.

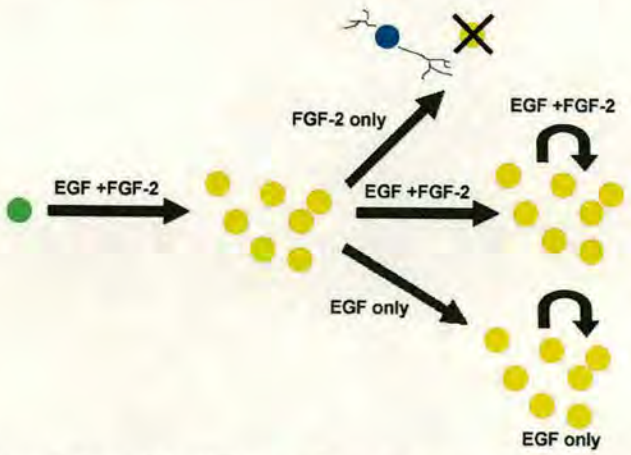

Figure 5. Roles of EGF and FGF-2 in the derivation and maintenance of NS cells. Fetal forebrain progenitors or ES cell–derived neural progenitors (left, green) can be converted into NS cell lines (yellow) using a combination of EGF with FGF-2. Once established, NS cells can be maintained in added EGF alone, whereas in FGF-2 alone, they undergo differentiation (blue) and apoptosis.

Notes

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Niche-Independent Symmetrical Self-Renewal of a Mammalian Tissue Stem Cell

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Pluripotent mouse embryonic stem (ES) cells multiply in simple monoculture by symmetrical divisions. In vivo, however, stem cells are generally thought to depend on specialised cellular microenvironments and to undergo predominantly asymmetric divisions. Ex vivo expansion of pure populations of tissue stem cells has proven elusive. Neural progenitor cells are propagated in combination with differentiating progeny in floating clusters called neurospheres. The proportion of stem cells in neurospheres is low, however, and they cannot be directly observed or interrogated. Here we demonstrate that the complex neurosphere environment is dispensable for stem cell maintenance, and that the combination of fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) is sufficient for derivation and continuous expansion by symmetrical division of pure cultures of neural stem (NS) cells. NS cells were derived first from mouse ES cells. Neural lineage induction was followed by growth factor addition in basal culture media. In the presence of only EGF and FGF-2, resulting NS cells proliferate continuously, are diploid, and clonogenic. After prolonged expansion, they remain able to differentiate efficiently into neurons and astrocytes in vitro and upon transplantation into the adult brain. Colonies generated from single NS cells all produce neurons upon growth factor withdrawal. NS cells uniformly express morphological, cell biological, and molecular features of radial glia, developmental precursors of neurons and glia. Consistent with this profile, adherent NS cell lines can readily be established from foetal mouse brain. Similar NS cells can be generated from human ES cells and human foetal brain. The extrinsic factors EGF plus FGF-2 are sufficient to sustain pure symmetrical self-renewing divisions of NS cells. The resultant cultures constitute the first known example of tissue-specific stem cells that can be propagated without accompanying differentiation. These homogenous cultures will enable delineation of molecular mechanisms that define a tissue-specific stem cell and allow direct comparison with pluripotent ES cells.

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Introduction

Stem cells are capable of generating identical progeny through unlimited numbers of cell divisions whilst retaining the ability to respond to physiological demands by producing daughters committed to differentiate. In vivo, stem cells are thought to reside in specific cellular microenvironments, or niches, that constitute privileged settings for support of self-renewal [1–4]. In tissues that utilise stem cells to sustain cell turnover, the stem cell compartment must be renewed in balance with the production of transit-amplifying progenitors [5]. This requires either equivalence between symmetrical self-renewal and commitment divisions, or an asymmetric mode of stem cell division. Expansion of stem cells, in vivo or in vitro, unambiguously requires symmetrical self-renewal. However, with the notable exception of embryonic stem (ES) cells, it has proven extremely problematic to propagate homogenous cultures of stem cells ex vivo. Epidermal stem cells [6] and neural stem cells [7] can be expanded in vitro, although accompanied by differentiation. It is unclear whether this reflects a dependence of tissue stem cells on a cellular niche, an intrinsic bias of tissue stem cells towards asymmetric division, or a failure to develop appropriate culture conditions to suppress commitment and sustain symmetrical self-renewal, as has been achieved for ES cells [8].

Neural stem cells appear to be sustained in a complex niche in the mammalian brain [9–11]. In 1992, Weiss and Reynolds made the landmark discovery that neural stem cells could be maintained in culture via propagation of floating cell clusters termed "neurospheres" [7]. Neurospheres consist predominantly of committed progenitors mixed with differentiated astrocytes and neurons. This mixed cellular environment likely provides a niche that sustains relatively few stem cells [12]. The neurosphere assay has proven invaluable in demonstrating the potential to give rise to stem cells in the developing and adult central nervous system (CNS) of rodents and primates [13–15]. However, neurospheres have significant limitations. The stem cells maintained within neuro-

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Abbreviations: BLBP, brain lipid binding protein; CNS, central nervous system; E, embryonic day; EGF, epidermal growth factor; ES, embryonic stem; FGF-2, fibroblast growth factor 2; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; HVA, high-voltage activated; LVA, low-voltage activated; MAP2, microtubule associated protein-2; NS, neural stem; P, postnatal day

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spheres are not directly identifiable, have not been purified, and have an uncertain relationship to CNS precursor cells in vivo [16]. Cellular complexity is a barrier to molecular and biochemical dissection of self-renewal and commitment mechanisms [17]. Heterogeneity also undermines comparative analytical approaches such as global expression profiling [16]. Furthermore, there is variation between as well as within cultures, which can give rise to contradictory data from different laboratories [18]. Finally, neurospheres differentiate much more readily into astrocytes than neurons in vitro [18] and in vivo [19], providing little enthusiasm for pharmacological screening or therapeutic applications [20]. Neural progenitor cells are also propagated in adherent cultures supported by fibroblast growth factor 2 (FGF-2) [21,22], but without genetic transformation [23,24], neuronal differentiation potential is usually progressively lost in these conditions [25,26]. As in *Drosophila*, mammalian neural progenitor cells may undergo asymmetric divisions in vivo [27,28] and in vitro [29]. However, the incidence of asymmetric versus symmetric division in true stem cells, either in vivo or in neurospheres, is unknown. Here we have investigated the potential for symmetrical self-renewal of neural stem cells and maintenance of neuronal differentiation capacity in fully defined adherent cultures.

Results

Derivation of Self-Renewing Adherent Neural Stem Cells from ES Cells

Mouse ES cells differentiate efficiently into neural precursor cells upon withdrawal of serum in adherent monolayer culture [8] or via treatment of embryoid bodies with retinoic acid [30,31]. These precursors have previously been expanded in FGF-2 with transient retention of neuronal differentiation potential [31,32], but continuous propagation is accompanied by restriction to glial fates ([33] and unpublished data). A similar switch from neurogenic to gliogenic differentiation is consistently observed in adherent cultures of primary foetal progenitors and is suggested to recapitulate the developmental progression during formation of the nervous system [25,34].

We induced neural precursor differentiation from ES cells in serum-free adherent monoculture [35,36]. After 7 d, cells were re-plated in basal medium (NS-A plus N2) in the presence of either FGF-2 alone or FGF-2 plus epidermal growth factor (EGF). Importantly, NS-A media does not support propagation of residual undifferentiated ES cells and they are thereby eliminated from the cultures. Neural precursors initially associate into floating clusters in this media. After 3–5 d, these aggregates were harvested, separating them from any adherent differentiated cells, and re-plated in fresh medium. They attached within 2–3 d and outgrew a population of bipolar cells. Upon passaging, these cells did not persist in FGF-2 alone, but in FGF-2 plus EGF they proliferated in the absence of other cell types. These bipolar cells, named LC-1, can be continuously and rapidly propagated with a doubling time of approximately 24 h.

LC1 cells express the immature neural marker nestin and are immunoreactive with the RC2 antibody, which recognises neural precursors, but expression of the astrocyte differentiation marker glial fibrillary acidic protein (GFAP) or of neuronal antigens is negligible (Parts b, c, and d in Figure 1A).

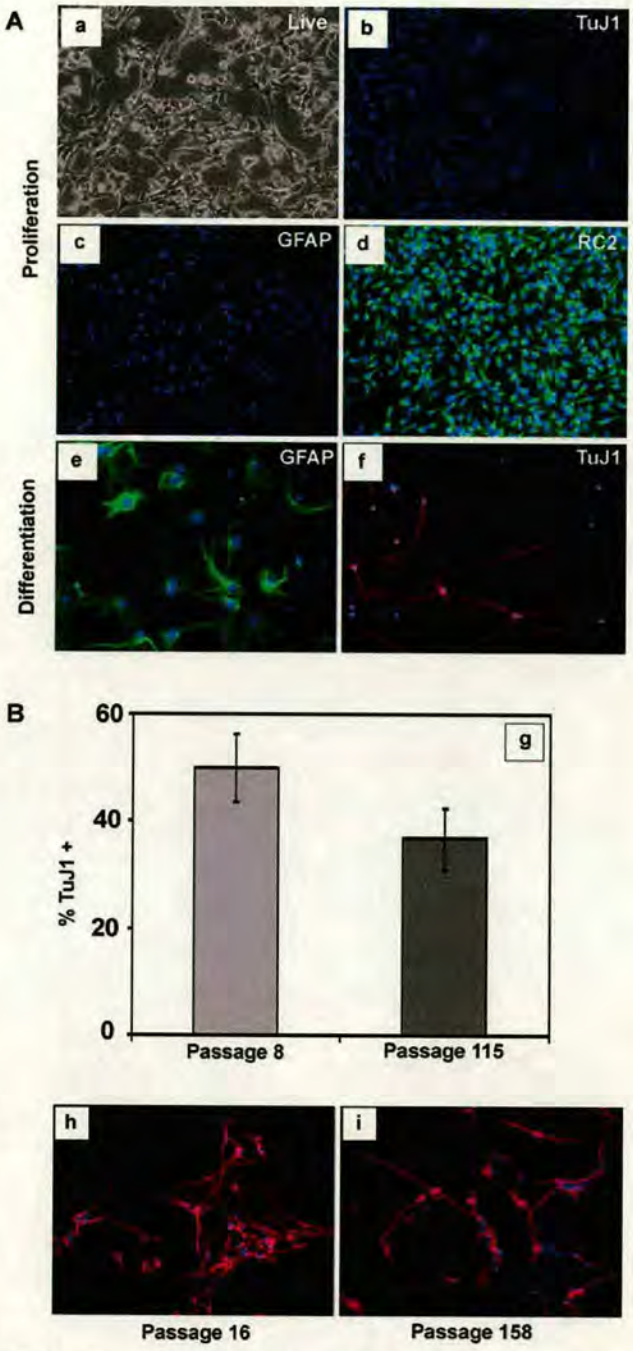

Figure 1. Generation of NS Cells from ES Cells

(A) The adherent NS cell culture (LC1) propagated in EGF and FGF-2 (a), shows no expression of neuronal (b) or astrocyte (c) antigens, and uniform expression of the precursor marker RC2 (d) and nestin (not shown). LC1 cells differentiate into GFAP immunopositive astrocytes (e) upon addition of serum and generate TuJ1 immunopositive neurons (f) upon growth factor withdrawal.

(B) The proportion of neurons obtained remains greater than 35% of total cells after 115 passages (g). Immunostaining for MAP2 of LC1 differentiation shown at passage 16 (h) and passage 158 (i).

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Upon exposure to serum or BMP 4, LC1 cells adopt astrocyte morphology within 48 h and subsequently uniformly express GFAP (Part e in Figure 1A). In contrast, cells with fine extended processes appear after re-plating on laminin

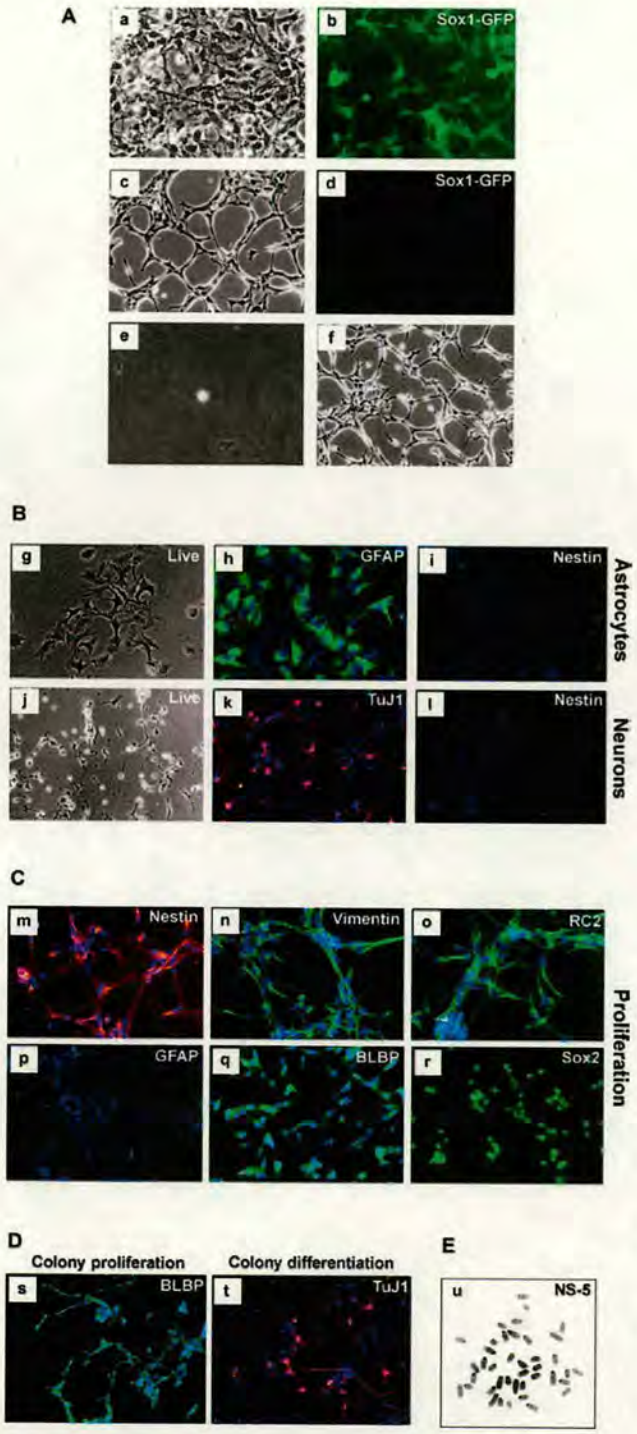

Figure 2. Clonal NS Cells Generated through *Sox1* Neural Lineage Selection

(A) Phase image of neural precursors at passage 1 (a) and 5 (c), with (b) and (d) showing corresponding *Sox1*-GFP fluorescence. Image (e) shows a single cell, 1 h after plating in Terasaki well, and (f) shows a phase-contrast image of clonal cell line at passage 20.

(B) Differentiation of NS-5 cells into astrocytes (g,h) and neurons (j,k) with loss of nestin immunoreactivity (i,l).

(C) These NS-5 cells are immunoreactive for neural precursor cell/radial glia markers (m–o,q,r) and negative for GFAP (p).

(D) Clones of NS-5 cells exhibit homogenous expression of BLBP with no immunoreactivity for GFAP in the presence of EGF/FGF (s), and generate neurons upon growth factor withdrawal (t).

(E) Metaphase spread of NS-5 (passage 31).

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without EGF for 5–7 d and then withdrawing FGF-2. These cells express neuronal markers type III β-tubulin (Part f in Figure 1A), microtubule associated protein-2 (MAP2) (Figure 1B), and neuN (not shown). High numbers of such neuronal cells, between 30% and 40% of total surviving cells, are generated with no significant decline even after 115 passages (Part g in Figure 1B). Together with the observation that LC1 cells retain diploid chromosome content at late passages (unpublished data), these data suggest the presence of self-renewing neural stem (NS) cell cultures.

NS cells competent for both glial and neuronal differentiation have subsequently been obtained using the procedure described for LC1 from more than ten ES cell lines, originating from three independent derivations: E14, CGR8, and R1. For all NS lines examined more than 95% of cells express nestin and are immunoreactive with RC2 in the presence of FGF-2 plus EGF. These NS cells are transfectable by electroporation (unpublished data) and can reliably be recovered from standard cryopreservation.

To assess whether the serum-free adherent neural induction protocol is a prerequisite for NS cell generation, ES cells were induced to differentiate by embryoid body formation and exposure to retinoic acid in serum-containing medium [30]. Aggregates were subjected to Sox2-βgeo lineage selection with G418 [31,37] for 48 h to enrich for neural precursors, then dissociated and cultured in the presence of FGF-2 and EGF without serum. Floating clusters formed that subsequently attached and outgrew Sox2-positive, nestin-positive, proliferative cells, which displayed the bipolar morphology and lattice growth typical of NS cells, as well as the capacity for astrocyte and neuronal differentiation after multiple passages (unpublished data).

Clonogenic NS Cells Derive from Sox1-Positive Pan-Neural Precursors

To investigate the origin of NS cells and to determine whether the floating cluster phase is essential for their generation, we induced neural commitment in monolayer and then maintained the neural precursors in N2B27 medium, in which condition they remain adherent [38]. To eliminate undifferentiated ES cells and non-neural differentiation products under these conditions, we again exploited lineage selection [31]. In this case we used 46C ES cells in which the green fluorescent protein (GFP)irespac reporter/selection cassette is integrated into the *Sox1* gene, an early marker of neural specification [39]. Transient puromycin selection after differentiation induction yields a purified population of neural precursors with minimal residual ES cells [40] (Parts a and b in Figure 2A). Either FGF-2 alone or FGF-2 plus EGF were then applied to the Sox1-expressing neural precursors maintained in N2B27 medium. Appreciable numbers of bipolar cells of NS morphology appeared only in the presence of both factors (Part c in Figure 2A). Initial heterogeneity of the population reduced with two to three passages. as astrocytes and other cell types decreased in number. Notably, expression of Sox1-GFP (and endogenous

Sox1) is lost during this process (Parts c and d in Figure 2A), but the cells remain positive for Sox2 and nestin. As they began to dominate the cultures, the bipolar cells formed extensive lattices. To establish the presence of clonogenic NS cells, single cells were isolated in microwells and expanded as adherent cultures (Parts e and f in Figure 2A). Five clonal lines were derived with morphology and growth characteristics similar to the bulk population. Initially one clone, NS-5, was characterised in detail, but subsequently all essential features described below were confirmed for other clones. These cells lack detectable expression of the pluripotency factors Oct4 and Nanog, and also of the early neural marker Sox1, but retain the pan-neuroepithelial marker Sox2 (Part r in Figure 2C). They are competent for astrocyte and neuronal differentiation (Figure 2B). Like LC1 cells, NS-5 cells uniformly express nestin, RC2, and other neural precursor markers (Figure 2C), and lack detectable GFAP expression (Part p in Figure 2C). We conclude that NS cells can be generated through a transient Sox1-positive neuroectodermal precursor via continuous adherent culture.

To establish that the NS-5 clone can generate sub-clones of the same phenotype, cells were plated at clonal density in EGF plus FGF-2. Multiple colonies were generated, all consisting of bipolar cells. Every colony shows immunoreactivity with RC2 (not shown) and expression of brain lipid binding protein (BLBP) (Figure 2D) in virtually all cells, with no detectable GFAP (Part s in Figure 2D). Several colonies were picked and expanded over ten or more passages with retention of these characteristics. Every colony can also be induced to differentiate into neurons (Part t in Figure 2D). Finally, we prepared metaphase spreads from NS-5 and determined a modal chromosome count of 40 (Figure 2E). The NS-5 clone therefore represents a clonogenic, genetically stable, NS cell line that self-renews continuously without requirement for a specialised cellular niche.

Contributions of EGF and FGF to NS Cell Self-Renewal and Lineage Commitment

As outlined above, NS cells were derived by culture only in the combination of FGF-2 plus EGF. This contrasts with previous studies, including our own, which used FGF-2 alone, resulting, after several passages, in populations of glial restricted progenitors [31–33]. We examined whether NS cells remained continuously dependent on EGF. When EGF is withdrawn from the cultures, massive cell death ensues after 20 h (Figures 3A and 3B), and the few cells that survive adopt differentiated morphology. This cell death is associated with presence of activated caspase 3, indicative of apoptosis (Figures 3C and 3D). Serum or BMP override the cell death response and drive the NS cells into astrocytic differentiation. We conclude that EGF firstly allows the maintenance of stem cells with competence for neurogenesis in contrast to FGF-2 alone, and secondly supports self-renewal of NS cells, acting in part via suppression of apoptosis.

We found that laminin could preserve cell viability in the absence of EGF. Simultaneous removal of EGF and FGF-2 on laminin results in astrocyte differentiation (unpublished data). However, NS cells cultured on laminin with FGF-2 alone do not express GFAP. Instead, they develop more extended processes (Figures 3E and 3F), and slow their rate of cell division. To date, we have been unable to maintain proliferation upon dissociation and passaging using FGF-2,

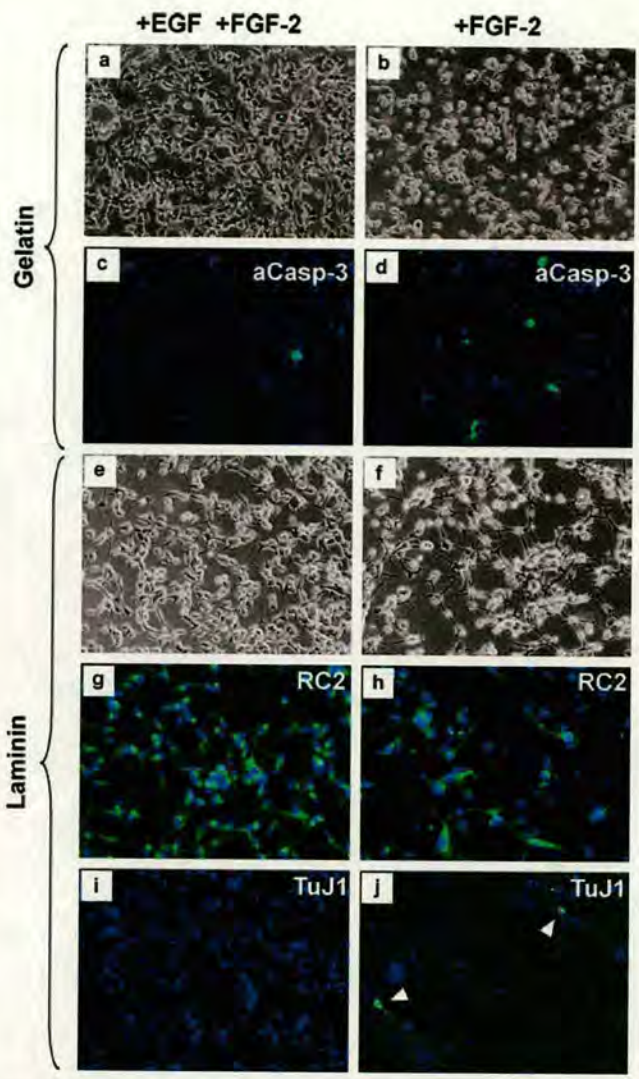

Figure 3. NS Cells Die or Begin to Differentiate in the Absence of EGF

Unlike proliferating cultures in FGF plus EGF (A,C), NS cells on gelatin die by caspase-3-mediated programmed cell death 20 h after removal of EGF (B,D). This death can be overcome if cells are cultured on a laminin substrate in FGF-2 only (F). Under these conditions, cells become slow-dividing and extend longer processes (G,H). Most cells retain RC2 immunoreactivity (H), but a minority begin neuronal differentiation marked by TuJ1 expression (J).

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and the cells die out when this is attempted. The majority of cells remain immunoreactive with RC2 in the presence of FGF-2, but TuJ1-immunostained immature neurons, which are never apparent in FGF-2 plus EGF, can be detected at low frequency (Figures 3G–3J). Upon subsequent withdrawal of FGF-2, the neuroblast marker doublecortin is expressed by a sub-population of cells (unpublished data). Many cells of neuronal morphology that express a diagnostic neuronal immunophenotype then appear (see Results), especially in presence of the neuronal viability supplement B27 [41]. These data suggest that upon release from EGF stimulation, the combination of laminin plus FGF-2 primes NS cells for neuroblast commitment with differentiation ensuing on mitogen withdrawal.

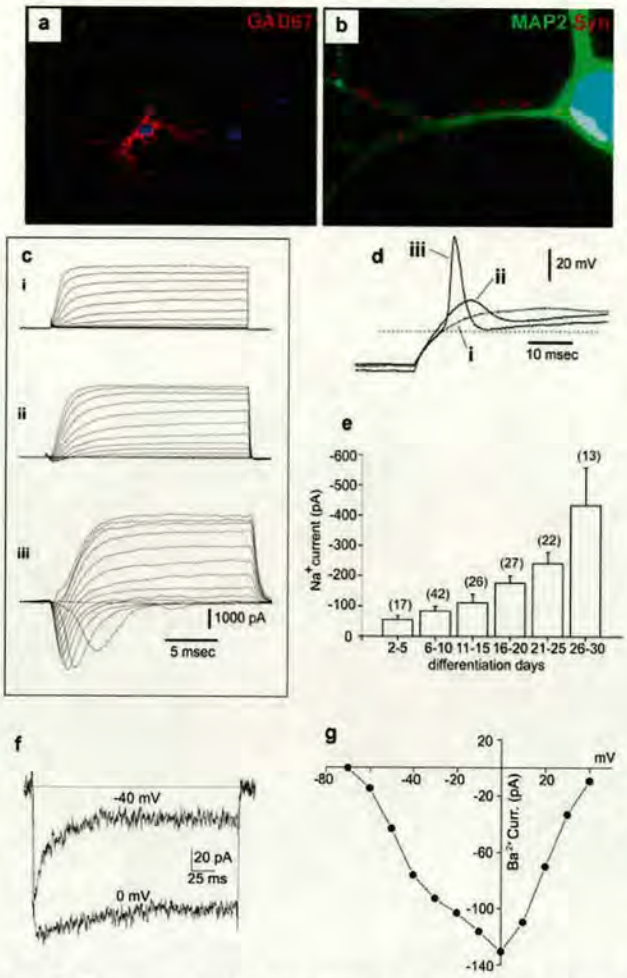

Figure 4. Phenotype and Electrical Activity of NS Cell–Derived Neurons

(A) This LC1 NS cell–derived neuron at 27 d of differentiation displays mature morphology and expresses GAD67.

(B) Expression of MAP2/synaptophysin after 7 d of differentiation.

(C) Superimposed inward and outward current tracings obtained at different membrane potentials (between −70 and +40 mV from a holding potential of −90 mV), from NS cell–derived neurons after differentiation for 6 (i), 20 (ii), and 30 d (iii).

(D) Superimposed voltage responses obtained following injection of depolarising rectangular current pulses in the same three cells (i–iii) by switching from voltage- to current-clamp immediately after current recordings shown in (c) were obtained. The dashed line represents a voltage level of −60 mV.

(E)Average Na^+ currents elicited at −20 mV from cells cultured in differentiating medium for increasing times as indicated by labels. Bars indicate SE.

(F) Superimposed inward currents elicited at −40 mV and 0 mV in 10 mM Ba^{2+} and in the presence of TTX; the holding potential was −90 mV.

(G) Current/voltage relationship from the same cell as in (F).

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Neuronal Differentiation of NS Cells

To assess the frequency of cells within NS cultures that are capable of neuronal differentiation, we plated NS-5 cells at clonal density on laminin, expanded for 12 d in EGF/FGF-2 followed by FGF-2 alone for 5 d, then a further 7 d in B27-supplemented media without growth factor. Every colony (126/126) produced TuJ1-positive cells (Part t in Figure 2D). These data indicate that all colony forming cells in NS cultures are competent for neuronal differentiation. Most neurons are immunopositive for GAD67 (glutamic acid decarboxylase) (Figure 4A) and gamma-aminobutyric acid (unpublished data) and by 7 d a sub-population shows expression of the mature marker synaptophysin (Figure 4B).

For unambiguous assignment of neuronal identity, we investigated the electrophysiological properties of differentiated NS cells. Figure 4C shows current recordings obtained during whole-cell voltage-clamp steps to depolarising test potentials. A sizeable outward voltage-gated current, with features of a delayed-rectifier K^+ current, is present by 6 d of differentiation (trace i). At later stages of differentiation (20 and 30 d, traces ii and iii), the current amplitude increases only slightly. By contrast, the amplitude of the inward current increases dramatically. Figure 4D shows the voltage responses elicited in the same cells after switching from voltage-clamp to current-clamp mode. The excitability properties of the cells correlate with the magnitude of the inward voltage-gated conductance. Thus, an overshooting action potential with a relatively fast depolarisation rate was elicited in the cell differentiated for 30 d (trace iii). The fast inactivating inward current was completely blocked by the selective Na^+ channel blocker tetrodotoxin (1 μM) and peaked at a test potential between −20 and −10 mV (unpublished data), typical features of voltage-gated Na^+ currents in neurons. The Na^+ current amplitude at −20 mV develops during differentiation (Figure 4E). The regenerative potential (ΔV measured between the threshold and the peak) elicited by the Na^+ current under current clamp conditions ranged between 0 and +20 mV during the first 15 d ($n = 6$), but after 25 d reached values between +30 and +70 mV ($n = 6$). Voltage-gated Ca^{2+} channel conductances were also detected (Figure 4F). The fast activating and relatively fast inactivating ($\tau_h = 21$ ms) current component elicited at −40 mV is reminiscent of the neuronal low-voltage activated (LVA) Ca^{2+} channel current [42]. By contrast, the Ba^{2+} current elicited at 0 mV, displaying a slow ($\tau_h = 73$ ms) and incomplete inactivation, has the typical features of the neuronal high-voltage activated (HVA) Ca^{2+} channel current. The presence in this cell of two distinct, LVA and HVA, Ca^{2+} channel conductances is confirmed by the current-voltage relationship (Figure 4G). On average, the LVA current peaked at −40 mV, while the I/V relationship for the HVA current peaked at 0 mV. A HVA Ba^{2+} current was detectable in 19 out of 27 cells, while a LVA current component was measured in 60% of the cells already expressing a HVA Ca^{2+} current ($n = 13$). In summary, NS cell–derived neurons are electrophysiologically active, exhibiting excitability properties and underlying voltage-gated Na^+ and Ca^{2+} conductances typical of maturing nerve cells.

NS Cells Exhibit Phenotypic Similarities to Radial Glia

Undifferentiated NS cells were then examined in more detail to gauge their developmental identity. By RT-PCR and Genechip analyses, they were found to lack pluripotency marker genes such as *Oct-4*, *nanog*, and *Eras* and markers of mesoderm or endoderm (Figure 5A and unpublished data). They express Pax6, Glast, and BLBP mRNAs (Figure 5A), and are immunopositive for nestin, RC2, vimentin, 3CB2, SSEA1/Lex1, Pax6, and prominin (Figures 5C, 5D, and S1). This set of markers is considered diagnostic for neurogenic radial glia, precursors of both neurons and astrocytes during development of the nervous system [43–45]. Retinoic acid treatment of ES cells has recently been shown to induce radial glia-like

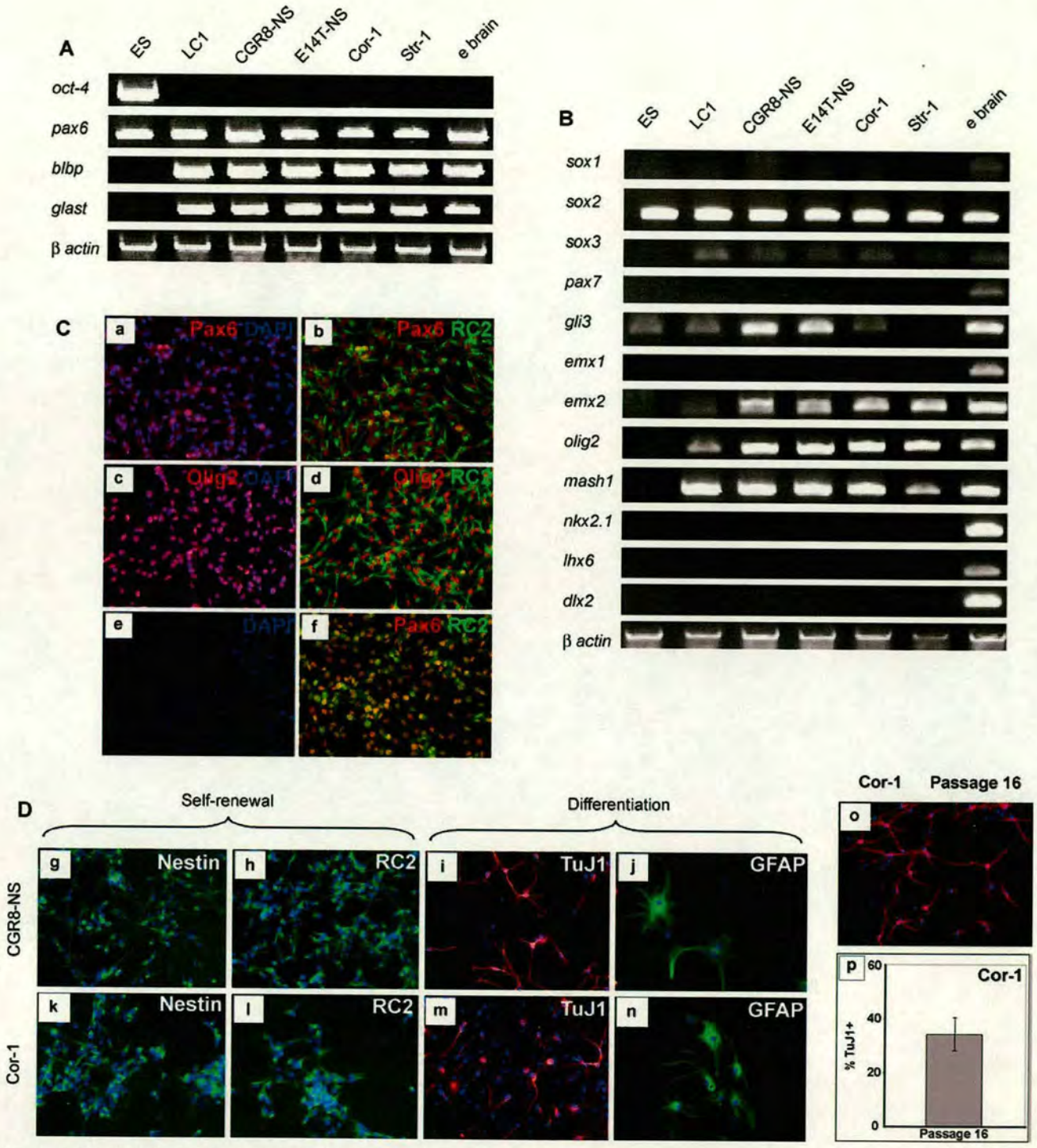

Figure 5. ES Cell–Derived or Forebrain-Derived NS Cells are Similar to Radial Glia

NS cells were derived from independent ES cell lines (CGR8, E14Tg2a) or primary cortical (Cor-1) and striatal (Str-1) tissue.
(A) RT-PCR of stem cell/radial glia markers.
(B) RT-PCR for pan-neural and region-specific transcriptional regulators.
(C) Double immunostaining for Pax6 and Pax6/RC2 (a,b), Olig2 and Olig2/RC2 (c,d) and Olig2/Pax6 (f). DAPI only for Olig2/Pax6 (e).
(D) The ES cell–derived line (CGR8-NS) and foetal cell–derived line (Cor-1) are indistinguishable from LC1 by morphology and NS cell/radial glial marker immunoreactivity (g,h,k,l), and can each differentiate into neurons (i,m) and astrocytes (j,n).
(E) The ability of Cor-1 to generate neurons (TuJ1+) is retained after 16 passages, more than 30 generations (p,o).
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cells as transient intermediates during neuronal differentiation [46,47]. Propagation of these radial glia cells was not described in these reports, however.

NS cells generally have elongated bipolar morphology, lamellate extensions, end-feet, and oval nuclei anticipated for radial glia [48]. Some more flattened cells and rounded cells with short extensions are also present. Immunostaining for the metaphase marker phosphorylated histone H3 indicates that the highly compacted cells are mitotic (unpublished data). Time-lapse videomicroscopy demonstrates a dynamic interconversion of morphology (Videos S1 and S2). In addition, time lapse reveals that NS cell nuclei undergo pronounced migration up and down the entire length of the cell process (Video S3). Such interkinetic nuclear migration is a well-characterised feature of neuroepithelial and radial glia cells in vivo [49]. It is striking to observe this occur in isolated cells, indicating that nuclear movement is a cell-autonomous property independent of cell–cell contacts or epithelial architecture.

All NS cells examined express the same panel of radial glia markers (Figures 5A and S2), plus the neural precursor markers Sox2, Sox3, and Emx2, and the bHLH (basic helix–loop helix) transcription factors Olig2 and Mash1 (Figure 5B). Although suggestive overall of telencephalic character, this set of markers does not neatly correlate with a specific regional identity. The presence of Olig2 and Mash1 is not characteristic of dorsal forebrain, and may reflect the ex vivo environment and a response to FGF-2. Indeed, *Olig2* has recently been found to be induced in spinal cord precursor cells and in neurospheres cultured in FGF-2, indicative of a relaxation of developmental specification [50,51]. Absence of Sox1, but maintenance of Sox2, is a noteworthy feature of NS cells, in view of the postulated determinative function of these transcription factors [52]. Whilst Sox1 marks all early neuroectodermal precursors, our data show that it is not retained in stem cells, where Sox2 may play the key role. NS cells also express Emx2, which is implicated in expansion of neural precursor cells [53,54] (Figures 5B and S1). Also, Dlx2, expressed in transit amplifying neuroblasts, but not stem cells in the sub-ventricular zone [55], is not detected in NS cells.

Most importantly, NS cells appear highly homogenous, staining uniformly for the various antigens examined. Double immunohistochemistry shows co-expression of Pax6/RC2, Olig2/RC2, Olig2/Pax6 (Figure 5C), and Pax6/Emx2 (Figure S1) in virtually every cell. Together with the absence of GFAP and TuJ1, this is indicative of pure symmetrical self-renewal divisions.

Derivation of NS Cells from Foetal Brain and from Neurospheres

ES cells are adapted for in vitro self-renewal [56], and this could in turn predispose for the propagation of derivative tissue stem cells. However, multiple phenotypic characteristics suggest that NS cells may be culture analogues of neurogenic radial glia. We therefore examined whether NS cell derivation depended on an epigenetic configuration carried over from ES cells or if they could be isolated from foetal neural tissue. Primary foetal CNS cells were harvested from embryonic day (E)16.5 mouse foetal forebrain and cultured in NS-A plus EGF/FGF-2. Initially, the cells adhered poorly to plastic and spontaneously formed floating clusters. After 6–7 d, these clusters were transferred to fresh dishes where they settled onto gelatin-coated plastic. Fourteen days later, outgrowths were trypsinised and re-plated. In three separate experiments, cells morphologically identifiable as NS cells proliferated and were subsequently expanded into continuous cell lines. These foetal brain derivatives express the same radial glia and neurogenic markers as the ES cell–derived NS cells (Parts k and l in Figure 5D, and Figure S2) and show consistent mRNA profiles by RT-PCR (Figures 5A and 5B) and by GeneChip analyses (unpublished data). They are likewise competent for neuronal and astrocyte differentiation (Parts m and n in Figure 5D). Cortex-derived Cor-1 cells were plated as single cells, and then colonies were subjected to sequential growth factor withdrawal as described for NS-5. Every colony produced TuJ1-positive neurons. This indicates that all clonogenic cells in the Cor-1 culture are neurogenic. These Cor-1 cells were also readily sub-cloned and continuously expanded from individual cells with retention of phenotypic markers of radial glia, as well as neuronal and astrocyte differentiation potential (Figure S2), indicative of self-renewal. Thus, NS cells derived from foetal brain share the key properties of ES cell–derived NS cells.

It has been reported that cells expressing radial glia markers persist in neurospheres [44] and that neurospheres can "differentiate" into radial glia [57]. It has also been shown that neurospheres can be obtained, albeit inefficiently, by continuous suspension culture during neural differentiation of ES cells [58]. We reasoned that NS cells may in fact be the resident stem cells within the neurosphere. Frozen/thawed, passage 40, mouse neurospheres derived from foetal forebrain were allowed to attach to gelatin-coated plastic in the presence of EGF and FGF-2. Bipolar cells outgrew that are indistinguishable from NS cells. These cells can be serially propagated as uniformly RC2-positive, GFAP-negative populations and then induced to differentiate into astrocytes or neurons (Figure S3). We conclude that radial glia-like cells present in neurospheres give rise to NS cells in adherent culture in the presence of FGF-2 plus EGF. Conversely, we observe that NS cells of either ES cell or foetal brain origin will readily form neurospheres, if detached from the substratum either mechanically or due to overgrowth. This suggests that NS cells/radial glia cells are likely the neurosphere forming stem cells. However, in contrast to adherent cultures, in neurospheres, stem cells constitute only a fraction of the cell population. This is presumably because aggregation induces differentiation, analogous to embryoid body differentiation by ES cell aggregates [59].

Survival and Differentiation of NS Cells Transplanted into the Rodent Brain

We investigated the behaviour of NS cells upon transplantation into mouse brain. ES cell–derived LC1 cells, transduced with a lentiviral enhanced GFP expression vector, were introduced into the developing brain by intra-uterine injection at E14.5 [60]. Animals were sacrificed after birth and the presence of GFP-positive cells was examined in brain sections. Mainly, GFP-labelled NS cell progeny migrated into striatum and cortex, with a few cells in the ventral telencephalon and olfactory bulbs. Immunohistochemical analyses revealed co-expression of enhanced GFP with the precursor marker nestin, neuronal markers TuJ, NeuN, and MAP2, and in lesser numbers with GFAP (Figure S4). NS cells were also injected into the adult mouse hippocampus and

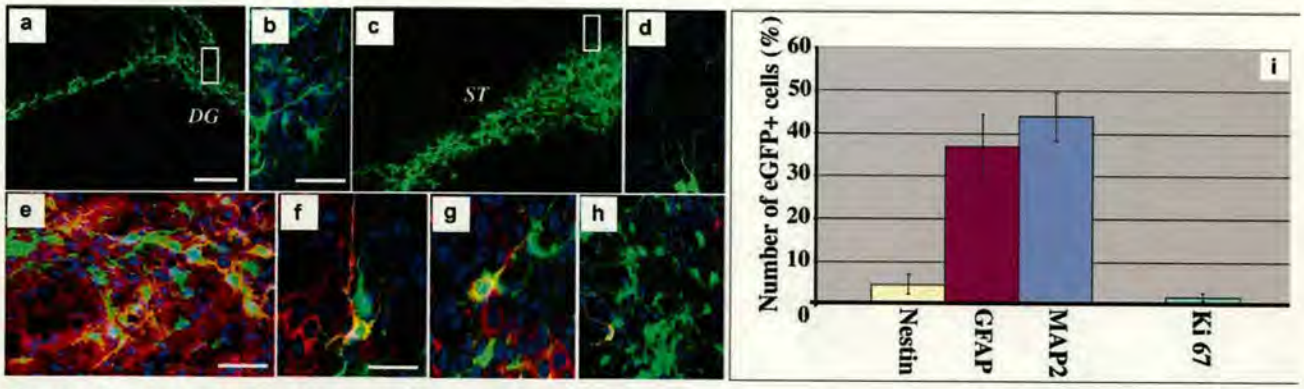

Figure 6. NS Cells Incorporate and Differentiate within the Adult Brain

(A–H) Confocal images of LC1 NS cells, lentivirally transduced with enhanced GFP, 4 wk post-grafting into hippocampus (A,B) or striatum (C–H). (B) and (D) show higher magnification of the insets in panels (A) and (C), respectively. Examples of enhanced GFP grafted NS cells (green) showing co-expression (yellow) of the neuronal markers TuJ (E, red) or MAP-2 (F, red), astroglial marker GFAP (G, red), neural progenitor marker nestin (H, red). (I) Quantitative analysis of graft-derived neuronal (MAP2), astroglial (GFAP), progenitor (Nestin), and proliferating (Ki67) cells, 4 wk after transplantation into adult mouse striatum. Data are means (± standard deviation) of at least 500 enhanced GFP+ cells from five independent animals. DG, Dentate Gyrus; ST, Striatum. Scale bars: A,C, 100 μm; B, D, E, 40 μm.; F–H, 20 μm.

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striatum. In this case, GFP-positive cells remained localised to the vicinity of the injection site. Four weeks after grafting, 44.4 ± 5.7% of GFP-expressing cells had neuronal morphology and were immunopositive for MAP2, 37.4 ± 6.1% expressed GFAP, and 4.2 ± 1.9% retained expression of nestin (Figure 6). A fraction of cells showed weak expression of either GFAP or neuronal markers that did not justify definitive classification but may be indicative of early stages of differentiation. The proliferative marker Ki67 was detected in only 1.0 ± 0.6% of GFP-positive cells, indicating that NS cells withdraw from the cell cycle in vivo. Well-differentiated GFP-labelled cells are also readily detected by GFP immunostaining in animals sacrificed 15 wk ($n = 4$) and 6 mo ($n = 4$) after grafting. We observed no histological evidence of unregulated proliferation or tumour formation in a total of 43 brains examined from 1–6 mo after transplantation, all with donor contributions. Furthermore, NS cells grafted to mouse kidney capsules did not proliferate or give rise to teratomas. These data indicate that NS cells can survive and differentiate in both foetal and adult brain environments and, unlike ES cells [61], they do not give rise to teratomas. Moreover, the relatively high frequency of neuronal differentiation is in contrast to grafts of passaged neurospheres, which appear predisposed in favour of glial differentiation [19,62]. The latter may reflect the incidence of committed glial progenitors present in neurospheres and seemingly absent in NS cell cultures

Derivation of NS-Like Cells from Human ES Cells and Foetal Cortex

Finally, we investigated whether similar NS cells could be isolated from human sources. In the process of attempting to derive human ES cells from donated supernumerary embryos, we observed, after 5–6 wk of culture, extensive spontaneous differentiation into rosette structures typical of neuroepithelial cells (Parts a and b in Figure 7A). These colonies were manually transferred into feeder-free culture in NS expansion medium. After a further 3–4 wk, bipolar cells similar to NS cells emerged from these cultures (parts c and d in Figure 7A) and have been continuously cultured for 5 mo. We also sourced Carnegie stage 19–20 human foetal cortex from elective terminations. Following tissue dissociation, cells initially formed floating aggregates that after 7 d were re-plated and allowed to attach to gelatin-coated plastic as for derivation of NS cells from mouse foetal brain (Parts i and j in Figure 7B). Proliferating cultures were established (Part k in Figure 7B). Human cultures derived from either ES cells or foetal forebrain were characterized by the presence of flattened cells associated with the bipolar cells. However, all cells express immature precursor markers nestin, vimentin, and 3CB2 (Figures 7A and 7B). Time-lapse monitoring confirmed that the two cell morphologies are plastic and interconvertible (Video S4). These human cells exhibit moderate levels of GFAP unlike mouse NS cells, but consistent with the known activity of the human GFAP promoter in radial glia [48,63]. They proliferate more slowly than the mouse cells, with doubling times of 5–10 d. After sequential withdrawal of EGF and FGF-2, they generate mixed populations of TuJ1-positive neuron-like cells and GFAP-positive cells (Part q in Figure 7C). Near-pure populations of cells with typical astrocyte morphology and intense GFAP immunoreactivity are readily produced after exposure to serum (Part r in Figure 7C). These data suggest that NS cells may be obtained from human as for mouse, although species-specific refinements may be required for optimal propagation and differentiation.

Discussion

The findings reported here establish that the growth factors EGF and FGF-2, plus insulin provided in N2, are sufficient to sustain robust expansion of neural stem cells in defined monoculture, liberated from any requirement for a specific cellular niche. Previous propagation of mammalian CNS precursor cells [64] has predominantly relied on short-term progenitor cell cultures [22,25], genetic immortalization of progenitors [23], or maintenance of stem cells within neurospheres [7]. Progenitor cells from the adult rat hippocampus have been propagated in adherent culture with FGF-2 whilst retaining ability to form neurons [65], albeit at low

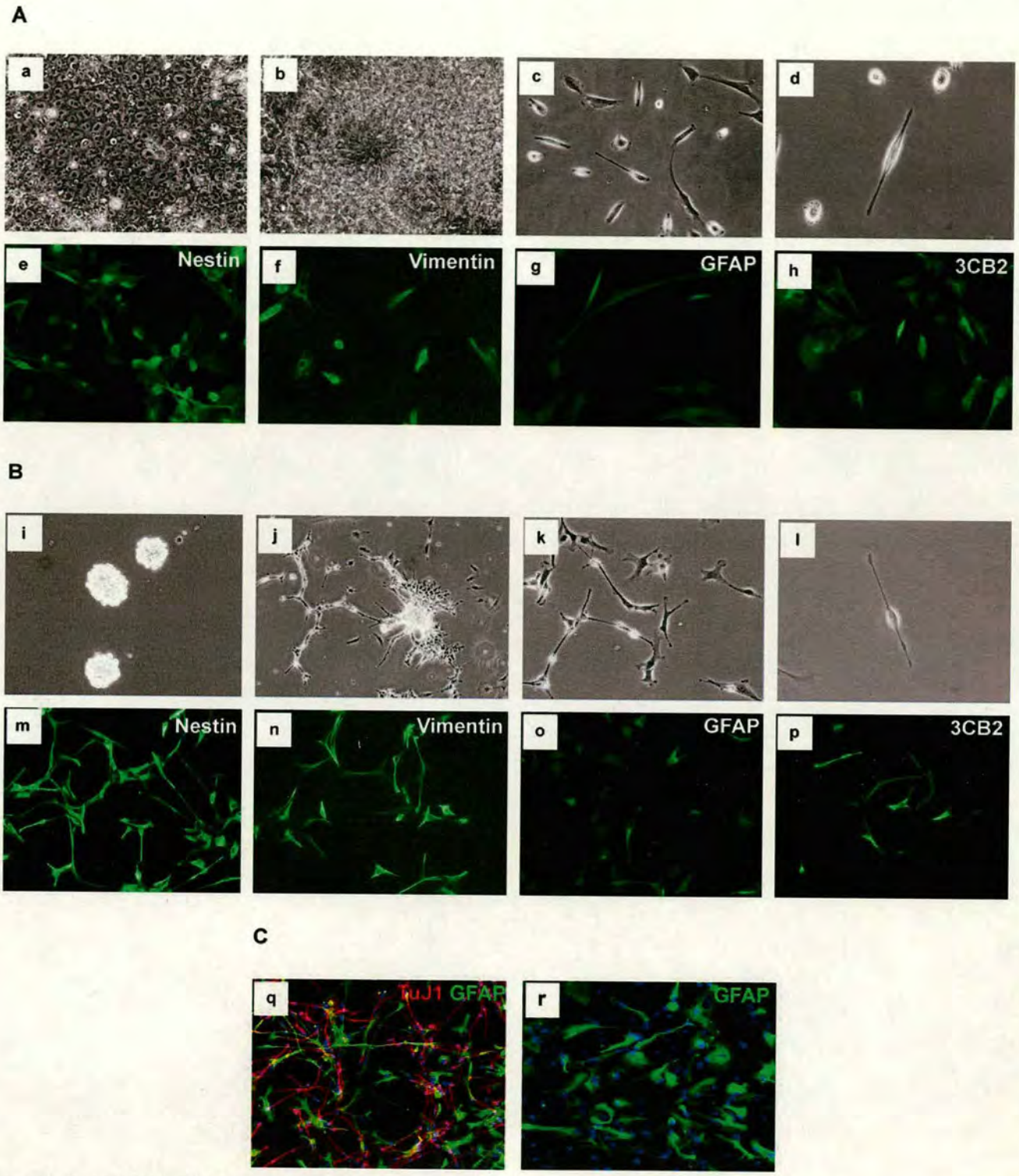

Figure 7. Human ES Cell or Foetal-Derived NS Cells

(A) Derivation from human ES cells: human ES cell primary culture (a), differentiation of human ES cells into neural-rosette structures (b), passage 9 in NS expansion medium (c), individual cells exhibit radial glial morphology (d), and immunostaining for NS cell/radial glia markers (e–h).
(B) Derivation from human foetal forebrain: floating clusters (i) generated from cortex, attachment and outgrowth (j), passage 5 in NS expansion medium (k), radial glia morphology (l), and NS cell/radial glial markers (m–p).
(C) Differentiation of human foetal NS cells: TuJ1 positive neuronal cells generated by sequential growth factor withdrawal (q), and GFAP positive astrocytes induced by exposure to serum (r).
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efficiency without inductive astrocytes [66]. These hippocampal-derived cells are heterogeneous by immunohistochemical staining and "undergo asymmetric cell divisions to continually replenish the supply of multipotent progenitors" [65]. In contrast, the uniformity and stability of marker expression in NS cells, combined with the long-term retention of neuronal differentiation efficiency, indicate that differentiation is completely suppressed by FGF-2 plus EGF in adherent culture. Consequently, the NS cells undergo continuous symmetrical self-renewal divisions.

The critical difference between the adherent culture system we describe and those used by others appears to be the use of EGF in addition to FGF-2. Although often applied to neurosphere cultures, EGF is typically omitted for attached cells. We find that continuous provision of EGF is essential for the derivation and propagation of NS cells, whether sourced from mouse ES cells or foetal brain. This may be related to the role of ErbB signalling in establishment of radial glia in vivo [67] and crosstalk with the Notch pathway [68]. Also, EGF functions to suppress apoptosis in NS cells (Figure 4B and 4D).

The evidence suggests that NS cells are closely related to a radial glia lineage. Whether any radial glia actually function as self-renewing stem cells in vivo is uncertain, since they exist only transiently during foetal development. However, recent fate mapping analysis [69] indicates that a subset of radial glia give rise to the sub-ventricular zone astrocytes that function as adult neural stem cells [70,71]. A possible relationship has also been proposed between hippocampal progenitors and radial glia [72]. The derivation of NS cells may reflect this intrinsic potential of radial glia to convert into stem cells. EGF has been reported to down-regulate expression of Dlx2 in transit-amplifying cells of the sub-ventricular zone and promote their conversion into neurosphere forming cells [55]. Consistent with this, we find that Dlx2 is not expressed in NS cells. Intriguingly, evidence has recently been presented that the EGF receptor undergoes asymmetric segregation in a proportion of RC2-staining neural progenitor cell divisions in vivo [28]. The retention or loss of the EGF receptor is suggested to facilitate alternative fate choices. Retention by both daughters of full responsiveness to EGF may be a central aspect of NS cell propagation. This could represent a crucial divergence from the circumstances of radial progenitor cells in vivo that underpins self-renewal in vitro.

NS cells do not express pluripotent cell-specific transcription factors Oct-4 and Nanog, but show appropriate expression of neural genes and absence of mesoderm and endoderm-specific genes. Their close relationship to a defined endogenous neural precursor cell, radial glia, adds further interest. NS cells can be cryopreserved, and may be transiently or stably transfected by electroporation or lipofection (unpublished data), or transduced with lentiviral vectors. They may be derived from previously engineered ES cells or transgenic mice or be genetically modified after derivation, opening new windows for genetic intervention into self-renewal and lineage commitment decisions in the nervous system and for investigation of neurodegenerative processes and oncogenic transformation. The potential of NS cells to generate different neuronal sub-types has yet to be determined, but their engraftment after transplantation into the adult brain suggests the potential for delivery of cell replacement and gene therapies. Whilst long-term stability and functional integration in vivo will have to be established in future studies, the preliminary data of human analogues to mouse NS cells provides encouragement for this approach.

In the context of fundamental stem cell biology, homogenous expansion of any stem cell in defined conditions has hitherto been the exclusive preserve of the ES cell. Ex vivo propagation of tissue stem cells has invariably been accompanied by differentiation, often interpreted as reflecting an intrinsic bias towards asymmetric division. The findings reported here show that this is definitively not the case for at least one class of neural stem cell. NS cells undergo sustained symmetrical self-renewal divisions with complete suppression of differentiation in response to FGF-2 and EGF. They thus provide a directly accessible system for molecular characterization and experimental manipulation of the stem cell state. Therefore, NS cells offer the first known tissue stem cell resource for direct comparison with ES cells in order to delineate common and distinct mechanistic features of lineage-restricted and pluripotent stem cells.

Materials and Methods

Mouse cell culture and differentiation. ES cells and neural differentiation are detailed elsewhere [38]. The LC1 and other ES cell–derived NS cells were routinely generated by re-plating d 7 adherent neural differentiation cultures (typically $2–3 \times 10^6$ cells into a T75 flask) on uncoated plastic in NS-A medium (Euroclone, Milan, Italy) supplemented with modified N2 [36] and 10 ng/ml of both EGF and FGF-2 (NS expansion medium). Over 3–5 d, cells formed aggregates that, after harvesting and sedimentation to remove debris, subsequently attached to fresh plastic and outgrew NS cells. After addition of 0.5 μg/ml of puromycin to differentiating adherent cultures at d 7, 46C-NS cells were generated. Cells were re-plated 3 d later into an uncoated T75 flask in N2B27 media with 10 ng/ml of both EGF and FGF-2 (Peprotech, Rocky Hill, New Jersey, United States) in the absence of puromycin. To derive clonal lines, including NS-5, single cells were plated into 96-well microwell plates (Nalge Nunc International, Rochester, New York, United States) by limiting dilution. and the presence of one cell per well was scored 1 h after plating.

For derivation directly from foetal CNS, primary cultures were generated using standard protocols from cortex or striatum of E16.5 mouse embryos and subsequently allowed to attach on flasks treated with 0.1% gelatin. Outgrowing cells were then expanded on gelatin using NS expansion medium. Clonal derivatives of the cortical line Cor-1 were established by plating at very low density (1,000 cells per 9-cm plate) and expanding individual colonies.

For derivation from established neurospheres, derived from E14 foetal brain and maintained for 40 passages in EGF plus FGF-2, cultures were dissociated to single cells using Accutase (Sigma, St. Louis, Missouri, United States) and plated at 10^4 cells/ml on gelatin-coated culture flasks in NS expansion medium.

For passaging established NS cell lines, we routinely used trypsin/EDTA or PBS and split cells 1:3 to 1:5 every 2–3 d. For astrocyte differentiations, NS cells were re-plated onto 4-well plates at 1×10^5 cells/well in NS-A medium supplemented with 1% fetal calf serum or 10 ng/ml BMP4 (R&D Systems, Minneapolis, Minnesota, United States). For neuronal differentiation, 5×10^4 NS cells were plated into poly-ornithine/laminin treated wells in NS-A supplemented with FGF-2 alone. After 7 d, the media was switched to NS-A supplemented with B27 (GIBCO, San Diego, California, United States) without growth factor. Half of the medium was exchanged every 2–3 d during the differentiation. For clonal differentiation, 1,000 cells from NS-5 or Cor-1, cultures were plated in 10-cm plates pre-treated with laminin, expanded for 12 d in EGF/FGF-2, and differentiated in situ as above. For electrophysiological studies, 1.5×10^5 NS cells were plated into poly-L-ornithine-treated 35-mm dishes in NS-A medium supplemented with N2 and B27 (both at 0.5%) and FGF-2 (5 ng/ml). After 7 d, the medium was switched to the mix NS-A:Neurobasal (1:1), supplemented with B27 (GIBCO) without growth factors. To sustain neuronal maturation, after a further 7 d, the medium was switched to the mix NS-A:Neurobasal (1:3) supplemented with B27 (GIBCO) and

brain derived neurotrophic factor (20 ng/ml) and nerve growth factor (R&D Systems; 50 ng/ml). Throughout neuronal differentiation, half of the medium was replaced every 2–3 d. Further details of NS cell derivation, propagation, and differentiation are provided in Protocol S1.

Characterisation of NS cells. Immunocytochemistry was performed using appropriate TRITC or FITC secondary conjugates and nuclear counterstaining with DAPI. Primary antibodies were used at the following dilutions: Nestin (1:10), Vimentin (1:50), Pax6 (1:5), 3CB2 (1:20), RC2 (1:50) (DSHB, Iowa City, Iowa, United States); TuJ (1:200) (Covance, Berkeley, California, United States); GFAP (1:300) (poly and mono, Sigma); MAP2 (1:200) (Chemicon, Temecula, California, United States; and Becton Dickinson, Palo Alto, California, United States); NeuN (1:200), gamma-aminobutyric acid (1:200), Gad65/67 (1:200) (Chemicon); Synaptophysin (1:200) (Sigma); Doublecortin (1:200) (Santa Cruz Biotechnology, Santa Cruz, California, United States), caspase-3 active (1:300) (R&D Systems), Olig2 (1:5000) (H. Takebayashi); Emx2 (1:2000) (A. Corte); BLBP (1:500) (N. Heins); prominin/mAb13A4 (1:200) (W. Huttner). Negative controls were ES cells, differentiated NS cells, or secondary alone. For RT-PCR, total RNA was extracted using RNeasy kit (Qiagen, Valencia, California, United States), and cDNA was generated using Superscript II (Invitrogen, Carlsbad, California, United States). PCR was performed for 30 cycles for all markers except β-actin (25 cycles). Details of primers and amplicon size are provided in Table S1. For metaphase spreads, cells were treated with 5 ml of 0.56% KCl for 20 min, fixed in methanol:acetic acid (3:1) on ice for 15 min, spread onto glass slides, and stained with TOPRO-3 (Molecular Probes, Eugene, Oregon, United States).

Solutions for electrophysiological and patch clamp recording. Recordings were made from LC1 cells differentiated between passages 20–25. Seals between electrodes and cells were established in a bath solution consisting of (in mmoles/l): 155 NaCl, 1.0 $CaCl_2$, 1 $MgCl_2$, 3.0 KCl, 10 glucose, and 10 HEPES/NaOH (pH 7.4). After establishing the whole-cell configuration, for current-clamp recording and for total current recording in voltage-clamp, the pipette filling solution contained (in mmoles/l): 128 KCl, 10 NaCl, 11 EGTA, 4 Mg-ATP, and 10 HEPES/KOH (pH 7.4). For the study of voltage-gated Na^+ channels under voltage clamp conditions, the patch pipette was filled with (in mmoles/l): 130 CsCl, 10 NaCl, 20 TEA-Cl, 10 EGTA, 2 $MgCl_2$, 4 Mg-ATP, and 10 HEPES/CsOH (pH 7.4), and the extracellular solution contained (in mmoles/l): 130 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 5 tetrethylammonium-Cl, $CdCl_2$ 0.2, and 10 HEPES/NaOH (pH 7.4). For the study of voltage-gated Ca^{2+} channels, the patch pipette was filled with (in mmoles/l): 120 CsCl, 20 TEA-Cl, 10 EGTA, 2 $MgCl_2$, 4 Mg-ATP, and 10 HEPES/CsOH (pH 7.4), and the extracellular solution contained (in mmoles/l): 130 NaCl, 10 $BaCl_2$, 10 glucose, 5 tetrethylammonium-Cl, 10 4-AP 1, TTX 10^{-3}, and HEPES/NaOH (pH 7.4). Ionic currents were recorded under voltage-clamp conditions using the patch-clamp whole-cell configuration at room temperature (20–24 °C) with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Burlingame, California, United States) and digitized at sampling intervals of 26–100 μsec using a Digidata 1322A A/D converter (Axon Instruments) interfaced with an IBM-compatible PC. Stimulation, acquisition, and data analysis were carried out using the software packages: pClamp 9 (Axon Instruments) and ORIGIN 6 (Microcal Software, Northampton, Massachusetts, United States). For voltage-clamp experiments, linear components of leak and capacitive currents were first reduced by analogue circuitry and then almost completely cancelled with the P/N method. Patch pipettes were made from borosilicate glass tubing and fire polished. Pipettes had a final resistance of 3–4 MΩ when filled with internal solution. Currents were filtered at 5 KHz.

Human embryo and foetal cultures. Research on human tissue with informed consent was approved by the Research Ethics Committee of Lothian Health Board. Frozen supernumerary human embryos were donated for research under licence R0132 issued by the Human Fertilisation and Embryology Authority. Inner cell masses were isolated by immunosurgery and cultured on human foreskin fibroblasts in medium supplemented with 15% serum replacement (Invitrogen) plus human leukaemia inhibitory factor and FGF-2 [73]. After three to four passages, cells with ES cell morphology differentiated into rosettes of neuroepithelial-like cells. These colonies were passaged into NS expansion medium without feeders or serum replacement. Human foetal tissue was obtained following elective termination with consent for research according to the Polkinghorne guidelines [74]. Cortex was dissected from Carnegie stage 19–20 foetuses and processed as described for mouse foetal tissue. In some cases, LIF (100 U/ml) was added to the expansion medium [75].

To induce neuronal differentiation, a similar protocol was followed as for mouse NS cells but with addition of brain derived neurotrophic factor (R&D Systems; 10 ng/ml) after the first 7 d without EGF, and retention of FGF-2 (5 ng/ml) until 14 d.

Transplants. To provide a convenient reporter, LC1 cells at passage 12 were transduced with lentiviral GFP. Transplants were performed after expansion of these cells for a further 9–28 passages. Foetal surgery was performed as described previously [60]. Using a glass capillary, 5×10^4 cells in a volume of 1 μl of HBSS were injected into the telencephalic vesicles of E14.5 Sprague-Dawley rat foetuses exposed under transillumination. Injected foetuses were replaced into the abdominal cavity for development to term. After delivery, animals were sacrificed at 7 d (postnatal day [P] 1, $n = 16$) and 5 wk (P30, $n = 8$) post-transplantation. For adult transplantations, 129 or CD1 mice were placed in a Kopf stereotaxic frame and received an injection of 2×10^5 NS cells suspended in 5 μl of HBSS into the striatum ($n = 22$) or hippocampus ($n = 21$). Transplanted mice were sacrificed after 2 ($n = 16$) and 4 wk ($n = 10$) and perfused transcardially with 4% paraformaldehyde. Cryosections (16 μm) were stained with the following antibodies: (mouse), NeuN (1:100) and Ki67 (1:10) (Chemicon), MAP2 (1:200; Becton Dickinson), Nestin (1:5; Ron McKay); (rabbit), βIII tubulin (1:500; Covance); GFAP (1:200; Dako, Glostrup, Denmark); secondary antibodies, Texas Red (Vector Laboratories, Burlingame, California, United States) (Jackson ImmunoResearch, West Grove, Pennsylvania, United States) and AlexaFluor 488 (Molecular Probes). Sections were preserved in antifading solution and analysed on Nikon TE2000-S ECLIPSE (Nikon, Tokyo, Japan) and Bio-Rad Radiance 2100 (Bio-Rad, Hercules, California, United States) confocal microscopes. Further cohorts of animals were sacrificed after 15 wk ($n = 4$) and 6 mo ($n = 4$) and analysed by antibody staining for GFP because the fluorescence signal was very low.

Supporting Information

Figure S1. Immunohistochemical Staining of NS Cells

(A) Radial glia/neural precursor markers: GLAST (Slc1a3) (a), prominin (b), and LeX/SSEA-1 (c), in NS-5 cells (equivalent results with LC1 not shown).

(B) Co-expression of transcription factors Emx2 and Pax6: DAPI (d), Emx2 (e), and Pax6 (f) double immunostaining (g, overlay) of LC1 NS cells (equivalent results with NS-5 not shown).

Found at DOI: 10.1371/journal.pbio.0030283.sg001 (292 KB PDF).

Figure S2. Mouse ES Cell–Derived and Foetal Cortex–Derived NS Cells Uniformly Express Radial Glia Markers

NS cells derived from CGR8 ES cells or from E16 foetal cortex (Cor-1 and clonal derivative Cor-1.3) were analysed for expression of the indicated markers by immunochemistry. Examination at high power shows that the radial glia markers are each expressed in almost all cells whilst they are uniformly negative for GFAP.

Found at DOI: 10.1371/journal.pbio.0030283.sg002 (641 KB PDF).

Figure S3. NS Cells Can Be Derived from Expanded Foetal Forebrain Neurospheres

NS line derived from a long-term foetal neurosphere culture (40 passages) exhibits identical morphology (a) to ES-derived NS lines, expresses neural precursor cell/radial glial marker immunoreactivity (b–d), and can differentiate into neurons (e) and astrocytes (f).

Found at DOI: 10.1371/journal.pbio.0030283.sg003 (284 KB PDF).

Figure S4. NS Cells Migrate and Differentiate after Transplantation in Foetal Rat Brain

Confocal image of NS cells, lentivirally transduced with enhanced GFP, 1 wk after transplantation into the ventricle of E14.5 rats (a). Donor cells migrate from the ventricle into the parenchyma in clusters and as single cells. Grafted cells show co-localization (yellow) of enhanced GFP (green) and; the neuronal marker MAP2 (b, red); astroglia marker GFAP (c, red) or; progenitor cell marker, nestin (d, red). Quantitative analysis (e) of graft-derived neuronal (NeuN and TuJ-1), astroglial (GFAP), progenitor (Nestin), and proliferating (Ki67) cells, one week after transplantation. Data are means (± standard deviation) of at least 500 enhanced GFP+ cells from five independent animals. LV, lateral ventricle. Scale bars: (a) 200 μm; (b–d), 20 μm.

Found at DOI: 10.1371/journal.pbio.0030283.sg004 (154 KB PDF).

Protocol S1. Derivation and Manipulation of NS Cell Lines

Found at DOI: 10.1371/journal.pbio.0030283.sd001 (59 KB DOC).

Table S1. Primers Used for RT-PCR

Found at DOI: 10.1371/journal.pbio.0030283.st001 (74 KB DOC).

Video S1. Mouse ES Cell–Derived (E14T-NS) NS Cells Show Dynamic Morphology (Low Magnification)

3 frames/second; 22 s running time.

Found at DOI: 10.1371/journal.pbio.0030283.sv001 (7.5 MB AVI).

Video S2. Mouse ES Cell–Derived (E14T-NS) NS Cells Show Dynamic Morphology (Higher Magnification)

3 frames/second; 72 s running time.

Found at DOI: 10.1371/journal.pbio.0030283.sv002 (4 MB MOV).

Video S3. Mouse Foetal Cortex–Derived NS Cell Exhibits Interkinetic Nuclear Migration

5 frames/second; 43 s running time.

Found at DOI: 10.1371/journal.pbio.0030283.sv003 (1.3 MB AVI).

Video S4 Human Foetal Cortex–Derived NS Cells Show Dynamic Morphology, Alternating between Bipolar and Spread-Out States

10 frames/second; 20 s running time.

Found at DOI: 10.1371/journal.pbio.0030283.sv004 (2.1 MB AVI).

Accession Numbers

The Swiss-Prot (http://www.ebi.ac.uk/swissprot) accession numbers for the genes and gene products discussed in this paper are *Ascl1* (Mash1) (Q02067), *Dlx2* (P40764), *Egf* (P01132), *Emx2* (Q04744), *Fabp7* (BLBP, B-FABP), (P51880), *Fgf2* (bFGF) (P15655), *Gad1* (Gad67) (P48318), *Gfap* (P03995), *Nanog* (Q7TN85), Nestin (Q6P5H2), *Olig2* (Q9EQW6), *Pax6* (P63015), *Pax7* (P47239), *Pou5f1* (Oct4, Oct3/4) (P20263), *Slc1a3* (Glast) (P56564), *Sox1* (P53783), *Sox2* (P48432), *Sox3* (P53784), and *Vim* (P20152).

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Competing interests. The University of Edinburgh has filed a patent application on methods of deriving and culturing neural stem cells relating to this study. This patent has been licensed to Stem Cell Sciences Ltd. AS holds non-voting equity (ca 5%) in Stem Cell Sciences Ltd.

Author contributions. LC, SMP,TG, EC, and AS conceived and designed the experiments. LC, SMP, TG, ER, MT, GB, YS, SS, QLY, and AS performed the experiments. LC, SMP, TG, ER, MT, GB, and YS analyzed the data. LC, SMP and AS wrote the paper. ∎

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